Development of a Protocol for the Proliferation of *In Vitro* Axillary Buds in Avocado (*Persea americana*) cv. 'Edranol'

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DECLARATION

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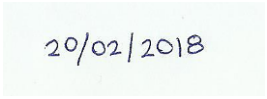

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ABSTRACT

Seed recalcitrance in avocado (*Persea americana*) has meant that avocado genetic material cannot be conserved in orthodox seed banks. Thus, biotechnological approaches have been considered for the long-term conservation of this species' genetic material, through the cryopreservation of tissue culture-generated axillary buds. A study was conducted to develop a system for the proliferation of *in vitro* avocado cv. 'Edranol' axillary buds for the purpose of cryopreservation.

Experiments were conducted to optimise avocado mother plant establishment and pretreatment. It was determined that potting soil mixes comprising of either 1:1:1 pine bark, perlite, river sand or 1:1:1 peat, perlite, river sand were suitable to culture healthy avocado mother plant seedlings. With these soil mixes approximately 2 shoots per plant developed after 11 weeks of transplanting and between 2.9 ± 0.31 and 3.37 ± 0.32 secondary shoots were produced after 5 months. Additionally, the mother plants produced well extended shoots (7.30 ± 1.29 cm; 8.77 ± 1.39 cm) with a sufficient number of axillary buds (7.75 ± 0.39; 6.33 ± 0.53), which were subsequently used as nodal explants. After surface decontamination, the establishment of an aseptic culture *in vitro* was successfully achieved.

Six semi-solid tissue culture media were tested for the proliferation of *in vitro* axillary buds. Four media comprised of half (½) and full strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), with either 0.5 or 1mg/l 6-Benzylaminopurine (BAP). Two media were based on the *P. indica* medium as proposed by Nel *et al.* (1983), and comprised of half strength MS macronutrients, full strength MS micronutrients, 2mg/l BAP and 1mg/l GA_3. All media were supplemented with 3g/l Gelrite and 30g/l sucrose at pH 5.6-5.8. Physiological measurements were taken six weeks after establishment, the first, the second and the third subculture.

Tissue browning, death and contamination were observed in explants cultured on the media containing 0.5mg/l BAP, suggesting that this concentration of BAP was not suitable for cv. 'Edranol'. Additionally, hyperhydricity appeared to be associated with the media containing ½ MS, which could be attributed to mineral deficiencies. Overall, there was no significant difference in the number of shoots and axillary buds developed across all the media tested, suggesting that endogenous auxin levels were higher than the concentration of cytokinin used in the media tested. In support of this, strong apical dominance and callus formation was observed. An increase in tissue browning, death and hyperhydricity on all the media tested,

coupled with a decrease in shoot length, suggested a decline in the vigour of explants *in vitro*. 1MS + 1mg/l BAP was selected as the most appropriate medium for the initiation of cv. 'Edranol' cultures, producing between 3.2 ± 0.2 and 4.9 ± 0.5 axillary buds per explant. However, hyperhydricity, browning and death were observed in explants cultured on this medium. Overall, the *in vitro* axillary bud explants did not behave predictably or uniformly. Thus, the system was not optimised, indicating that further study is needed for the mass multiplication of axillary buds to be used for the cryo-conservation of avocado genetic material.

It is recommended that future experiments will be needed to further test tissue culture media, with a focus on the optimisation of the nutrient and plant growth regulator concentrations. Additionally, the recalcitrance of explants to the *in vitro* environment may have been influenced by the physiological state of the mother plants, indicating that research should be focused on the effect which the mother plants may have on the endogenous responses of the *in vitro* explants.

Dedication: This dissertation is dedicated to my Beloved Parents and Brothers

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In the name of **Allah** (God), the Beneficent, the Merciful

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LIST OF SYMBOLS AND NOMENCLATURE

CPPU	1-(2-chloro-4-pyridyl)-3-phenylurea
NAA	1-naphthaleneacetic acid
BAP	6-Benzylaminopurine
NH_4NO_3	Ammonium nitrate
≈	Approximately equal to
BA	Benzyladenine
H_3BO_3	Boric acid
$CaCl_2$	Calcium chloride
cm	Centimetre
$CoCl_2.6H_2O$	Cobalt chloride hexahydrate
$CuSO_4.5H_2O$	Copper(II) sulphate pentahydrate
cm^3	Cubic centimetres
°C	Degrees Celsius
dS/m	DeciSiemens per meter
EC	Electrical conductivity
FeNaEDTA	Ethylenediaminetetraacetic acid ferric sodium salt
B5	Gamborg *et al.*(1968)
g	Grams
GA_3	Gibberellic acid
g/l	Grams per litre
HCl	Hydrochloric acid
IBA	Indole-3-butyric acid
$FeSO_4.7H_2O$	Iron(II) sulphate heptahydrate
$MgSO_4$	Magnesium sulphate
$MnSO_4.H_2O$	Manganese(II) sulphate
mg/l	Milligrams per litre
ml	Millilitre
ml/l	Millilitre per litre
mm	Millimetre
mM	Millimolar
$NaH_2PO_4.2H_2O$	Monosodium phosphate dihydrate
KH_2PO_4	Monopotassium dihydrogen phosphate
MS	Murashige and Skoog medium (1962)
No.	Number

%	Percent
% (v/v)	Percentage (volume per volume)
% (w/v)	Percentage (weight per volume)
PGRs	Plant growth regulators
PVP	Polyvinylpyrrolidone
KI	Potassium iodide
KNO_3	Potassium nitrate
$Na_2MoO_4.2H_2O$	Sodium molybdate dihydrate
SDW	Sterile distilled water
WPM	Woody plant medium (1981)
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

STRUCTURE OF THIS DISSERTATION

This dissertation in presented in five chapters. Chapter One contains a general introduction. Thereafter, Chapter Two details the initial experiments which were conducted to establish *in vitro* avocado material. Based on what was observed, after the initial experiments further study was needed to optimise the entire tissue culture system. Thus, Chapter Three details the experiments conducted to optimise the stage 0 preparations of the avocado mother plants. Following this, Chapter Four further explores avocado tissue culture (Stages 1 and 2). Finally, Chapter Five provides a general discussion and conclusion.

1 CHAPTER ONE - General Introduction

1.1.1 Background

Considering that access to food is central to human existence, the conservation of the genetic resources of crop species is essential in providing food security now and in the future (George, 2011). The most common *ex situ* method of plant germplasm conservation is through the storage of seeds in seed banks. Seed storage, however, is only suitable for the conservation of species that produce orthodox seeds. During their development, orthodox seeds dry to low water contents (5 to 10% water content) and become quiescent or dormant (Berjak and Pammenter, 2004). As such, orthodox seeds are tolerant to cold and dry conditions, and exposure to these conditions improves the longevity and viability of the seeds (Berjak and Pammenter, 2004). In contrast, recalcitrant seeds do not undergo maturation drying during their development and are shed from their parent plants while still highly hydrated and metabolically active (Berjak and Pammenter, 2002). Recalcitrant seeds are also sensitive to cold, and thus, cannot be stored in the cold and dry conditions of orthodox seed banks (Bonner, 2008).

This raises a conservation challenge as approximately 10% of angiosperm species produce recalcitrant seeds, including crop and economically-important species, such as tea, coffee and rubber (Walters *et al.*, 2013; Engelmann, 2011). In addition, many tropical fruit species, including avocado, litchi and mango produce recalcitrant seeds. To ensure that humans can obtain benefit from these resources in the future, alternative methods have been developed and used for the conservation of crop species that produce recalcitrant seeds.

1.1.2 Avocado the fruit crop species

Avocado (*Persea americana*), belonging to the angiosperm family Lauraceae, is a woody fruit tree species (Chanderbali *et al.*, 2013). The avocado is a semi-evergreen forest tree that is known to shed some of its leaves in spring (Wolstenholme *et al.*, 2013; Janick and Paull, 2008). Avocados can grow very large, often taller than 20 meters (Alcaraz *et al.*, 2013). Of the Lauraceae, avocado is the only species that produces an edible fruit - a large fleshy berry (Storey, 2008; Morton, 1987).

1.1.2.1 Distribution of avocado

Native to South America, today avocados are grown in most tropical and subtropical parts of the world (Morton, 1987). For commercial trade, avocados are most commonly grown in the United States, Mexico, Chile, Israel and South Africa (Ben-Ya'acov and Michelson, 1995; Morton, 1987). In South Africa, the major avocado production areas are the Limpopo (producing more than 50% of the national produce), KwaZulu-Natal and Mpumalanga provinces (DAFF, 2011).

1.1.2.2 Varieties of avocado

There are three main 'races', or subspecies, of avocado: the Guatemalan (*P. americana* var. *guatemalensis*), the West Indian (*P. americana* var. *americana*) and the Mexican (*P. americana* var. *drymifolia*) (Arias *et al.*, 2012; Bergh and Ellstrand, 1986). These races were categorised based on the area where they were believed to have originated (Bergh and Ellstrand, 1986). Within these races, there are many varieties of avocado, each with different properties in terms of fruit characteristics, climatic tolerances, fruiting times and growth forms (Janick and Moore, 1996). The Guatemalan race has most of the characteristics desirable for horticultural and economic purposes, including small seeds, oil content of 15-18% and nutty flavoured flesh with high nutritional value (Schaffer *et al.*, 2013b; Janick and Paull, 2008; Morton, 1987; Bergh and Ellstrand, 1986).

The most economically important varieties of avocado include the 'Hass', 'Fuerte', 'Pinkerton' and 'Ryan' (Schaffer *et al.*, 2013b; Paull and Duarte, 2011; Janick and Paull, 2008; Snijder *et al.*, 2003; Morton, 1987). The 'Edranol' variety is amongst the most commonly planted in South Africa for commercial purposes (SAAGA, 2012; Human, 1987; Bower *et al.*, 1977). This variety belongs to the Guatemalan race, and has characteristics representative of this race, including adaptation to a subtropical climate, intermediate cold tolerance and shade tolerance (Crane *et al.*, 2013; Bergh and Ellstrand, 1986; Bower *et al.*, 1977). This variety produces a medium size, pear-shaped fruit with a high oil content (Crane *et al.*, 2013; Morton, 1987). The seeds of this variety are used as nurse seeds in the double grafting of avocados (Ben-Ya'acov and Michelson, 1995). While this variety is considered to be disease resistant (Morton, 1987; University of California, Agriculture and Natural Resources, n.d.), it is an unsuitable source of rootstock, as it is susceptible to root rot disease (Snyman *et al.*, 1984).

1.1.2.3 Uses of avocado

The avocado fruit is amongst the most nutritious commercial fruits in the world (Wolstenholme *et al.*, 2013). Avocados are consumed worldwide, either fresh/raw, or are processed into oils. Avocados are a rich source of monosaturated fatty acids (oil), fibre, water, proteins and vitamins (A, C and B) (Wolstenholme *et al.*, 2013; Ding *et al.*, 2007). Other health benefits of avocado include that they are low in sodium, fat and calories (Ding *et al.*, 2007). Avocados are used for medicinal purposes, as they contain over 20 minerals and phytochemicals, which are being researched as a possible cure for different cancers (Ding *et al.*, 2007). The oils from avocados are also used in cosmetic and skin care products.

1.1.2.4 The economic importance of avocado

Due to the high quality nutritional value of the fruit, avocados are amongst the five most economically-important tropical fruit species in the world (DAFF, 2011; Wolstenholme *et al.*, 2013). The consumption of avocados has almost doubled in the last 10 years (Wolstenholme *et al.*, 2013). It is expected that as more countries export avocados, and more people become familiar with avocados, the production, consumption and trade of avocados will further increase (Wolstenholme *et al.*, 2013).

In 2012, the largest producers of avocado were Mexico (1.3 million tons), USA (245 thousand tons), Indonesia (224 thousand tons) and Chile (160 thousand tons) (FAOSTAT, 2014). Even though South Africa is not amongst the highest producers of avocado, producing 91 thousand tons in 2012, it is ranked among the five biggest exporters of avocado (FAOSTAT, 2014). In 2011, South Africa exported 30 thousand tons of avocado, with a value of R222 million (FAOSTAT, 2014). It is estimated that 45% of the total South African production is exported (of which 79% is exported to Europe), 15% is processed into oil and guacamole, 25% is sold in local markets and 15% is sold in informal markets (DAFF, 2011; Subtrop, 2009).

1.1.3 Propagation of avocado and germplasm conservation

The destruction of natural tropical habitats has led to a loss in the genetic diversity of wild and native avocado species (Barrientos-Priego *et al.*, 1995). Consequently, without conservation effort, potentially important genotypes which could be used in the breeding of avocado cultivars and their rootstocks will continue to be lost (Barrientos-Priego, 1999). In the following section, the methods of propagating avocados are discussed, with regard to the

limits that each method poses in terms of the long-term conservation of this species. As an alternative method tissue culture is considered.

1.1.3.1 Propagation by seed

The avocado tree produces an oval-shaped fruit, the size of which varies across varieties. Each fruit contains a single large pear-shaped recalcitrant seed, which continues to grow and germinate after being shed from its parent plant (Walters *et al.*, 2013). For commercial purposes, specific avocado genotypes with favourable traits are selected, and these genotypes are clonally propagated. Because seeds are a source of genetic variation, the propagation of avocado through seed is not suitable for commercial purposes.

1.1.3.2 Seed storage

The recalcitrant seeds of avocados cannot be stored in conventional seed banks as they are sensitive to desiccation and cold, and thus cannot be stored under the same conditions as orthodox seeds in seed banks (Bonner, 2008; Berjak and Pammenter, 2004, 2002). Additionally, avocado seeds become unviable within a month (Morton, 1987). Currently, the only method of storing recalcitrant seeds is 'wet storage', a process where seeds are stored at temperatures and relative humidities that allow for the water content of the seed to be maintained at the same level as it was when the seed was shed (Bonner, 2008; Berjak *et al.*, 1989). Wet storage, however, has many drawbacks. For instance, the conditions used in the wet storage of seeds promote microbial growth, and thus seeds become susceptible to microbial contamination (Mycock and Berjak, 1990; Chin *et al.*, 1989). Furthermore, avocado seeds are large, and would require a large amount of storage space. Consequently, the conservation of avocado germplasm cannot be achieved through seed storage.

1.1.3.3 Grafting

Avocados are most commonly vegetatively propagated (Ben-Ya'acov and Michelson, 1995). Varieties with desirable fruit characteristics are selected as scions, which are grafted onto varieties with disease resistant rootstocks (Ben-Ya'acov and Michelson, 1995). The problem with grafting is that seed-derived rootstocks exhibit genetic heterogeneity and thus are not suitable for commercial purposes, and more importantly, vegetative avocado rootstocks do not root easily (Bandaralage *et al.*, 2015; Barceló-Muñoz *et al.*, 1999; Cameron, 1955). This has led to the development of the Frolich and Platt technique (Frolich and Platt, 1972), which

is currently the most successful method of vegetatively propagating avocados. Using this method of double grafting, a section of the variety that is to be a future rootstock is grafted onto a seedling. Shoots that develop from this section are cut and the whole plant is placed in the dark to develop roots (Frolich and Platt, 1972). If a dark room is not available, a collar is placed around the stem of the plant. Once shoots begin to emerge, the collar is filled with vermiculite (Frolich and Platt, 1972). Thereafter, the shoots are placed in a shaded area until leaves develop. Once rooting has occurred, the grafted plant is severed from the seedling and is transplanted into an individual pot (Frolich and Platt, 1972). A scion of the fruiting variety is later grafted onto this rootstock (Frolich and Platt, 1972). Even though grafting is the most widely applied method of propagating avocados, there are still some limitations. For instance, grafted plants require specialised care and the process is time consuming (Hofshi, 1997).

In terms of conservation, for short term storage purposes, clonally propagated plants can be maintained in nurseries and plantations (Engelmann, 2004). However, this requires large amounts of land and maintenance, e.g. watering and weeding. In addition to this, plants are vulnerable to natural catastrophes, such as hail, frost and drought (Engelmann, 2004). Avocado plants in particular are susceptible to pests and diseases, particularly by *Phytophthora* species, a water mould causing root and fruit rot in adult avocado plants (Ploetz, 2005; Ben-Ya'acov and Michelson, 1995; Coffey, 1987), and do not respond well to soil stress, particularly salinity (Litz *et al.*, 2005). This means that even through grafting has been favoured for propagating avocados, the storage of grafted plants in nurseries is not without problems. It has thus become important to develop alternative propagation and storage techniques for avocado (Bowman and Scora, 1992).

1.1.3.4 In vitro propagation

In vitro propagation has been considered as an alternative method for the production of avocado (Hiti-Bandaralage *et al.*, 2017; Barceló-Muñoz and Pliego-Alfaro, 2012). *In vitro* propagation involves growing a plant in an artificial and controlled environment (George, 2008). There are many benefits to this approach, including that it is a disease free method, and it can result in the rapid generation of plant material (George, 2008; Razdan, 2003; Dodds and Roberts, 1985). Disadvantages of *in vitro* propagation include that it is costly and requires skilled personal (George, 2008; Razdan, 2003; Dodds and Roberts, 1985).

There are five stages in the mass propagation (micropropagation) of plants using *in vitro* methods (Table 1). In brief, during *in vitro* propagation, explants are aseptically removed

from their mother plants and cultured on an artificial medium (George and Deberg, 2008; Razdan, 2003). The growth medium usually contains macro and micro plant nutrients, a carbon source (most often sucrose), plant growth regulators and a gelling agent, which allows for the medium to solidify (George, 2008; Razdan, 2003; Dodds and Roberts, 1985). This growing medium may be species specific, and thus needs to be optimised experimentally for each species, and for genotypes within a species (Barceló-Muñoz and Pliego-Alfaro, 2012; George and Deberg, 2008; Razdan, 2003). In addition to this, other conditions that need to be optimised include the pH of the medium, culture photoperiod (duration and intensity) and temperature.

Table 1. Brief description of the five stages of *in vitro* plant propagation (George and Deberg, 2008; Razdan, 2003)

Stage of i*n vitro* propagation	Description
Stage 0: Mother plant selection and preparation	• Plants with typical characteristics of the species are selected • Plants are treated with systemic anti-microbial chemicals, to ensure that plants are almost disease free • Plants are supplied with nutrients that promote vigorous growth, to ensure that enough material is available for i*n vitro* establishment.
Stage 1: Establishing an aseptic culture	• Surface decontamination • Explants are plated onto a suitable sterile medium and are allowed to grow • Contaminated explants or media are discarded
Stage 2: Multiplication of suitable propagules	• Explants cultured on suitable medium under optimum conditions • Plantlets are divided and sub-cultured to increase numbers
Stage 3: Preparing plants for the natural environment	• *In vitro* plantlets are transferred to media that promote root development and shoot elongation

Stage 4: Transferring plants to the natural environment	• Plantlets are acclimatised before transfer to the greenhouse • Plantlets are transplanted from the medium to a suitable substrate, e.g. peat, loam

1.1.3.5 Storage of in vitro tissues

The development of ancillary methods has meant that *in vitro* tissues can be stored, which allows for the conservation and preservation of a species (Engelmann, 2011; Razdan, 2003; Benson, 2002). *In vitro* tissues are routinely sub-cultured onto fresh medium and the extension of the period between subcultures can be used for short-term storage (Engelmann, 2011; Razdan, 2003; Benson, 2002). However, this approach is labour intensive and time consuming, and haphazard maintenance of *in vitro* cultures may lead to contamination (Engelmann, 2011; Razdan, 2003; Benson, 2002). In addition to this, with constant sub-culturing *in vitro* tissues have a tendency to become less vigorous, and more prone to genetic mutations (Reed, 2004).

For medium term storage of *in vitro* tissues, slow growth techniques have been used. Slow growth techniques involve culturing *in vitro* plants on media or at temperatures that reduce growth (Shibli *et al.*, 2006; Watt *et al.*, 2000). Even through growth is slowed, metabolism still continues within cells, and genetic alterations can still occur (Shibli *et al.*, 2006; Watt *et al.*, 2000).

When considering the importance of conserving plant genetic material so that humans can still obtain benefit from it in the future, it is necessary to develop techniques for long term storage. The long term storage of plant genetic material can be achieved through cryopreservation, which is the storage of biological materials at sub-zero temperatures most often in or above liquid nitrogen (≈ -196°C) (Kaviani, 2011; Berjak and Pammenter, 2004; Withers and Engelmann, 1997; Mycock *et al.*, 1995; Engelmann, 1991). Cryopreservation can be used to conserve both specific genotypes (e.g. elite clonal crops), which have been selected for favourable qualities, and unknown genotypes (e.g. endangered indigenous species), for maintaining genetic diversity (Berjak *et al.*, 2011; Reed, 2008; Watt *et al.*, 2004). In contrast to medium term storage, during cryopreservation all metabolic activities within the plant tissues are reduced (Benson, 2002; Engelmann, 2011). Because of this, using

cryopreservation plant tissue can potentially be preserved indefinitely (Benson, 2002; Engelmann, 2011).

1.1.4 Understanding plant responses to the *in vitro* environment

It is important to develop an understanding of how plants respond to the *in vitro* environment, as tissue culture itself is a manipulation. The treatments that plants are exposed to in tissue culture may bring about characteristics of the plant that would not normally be seen in nature (Barceló-Muñoz and Pliego-Alfaro, 2012; Desjardins *et al.*, 2007; Razdan, 2003). Furthermore, *in vitro* manipulations may fundamentally alter the way plants respond to subsequent treatments (Barceló-Muñoz and Pliego-Alfaro, 2012; Desjardins *et al.*, 2007; Razdan, 2003). For instance, Nakhooda *et al.* (2011) observed that the type of auxin used during plantlet multiplication influenced rooting capacity and root morphology in *Eucalyptus grandis* clones. The present study was focused on developing an *in vitro* system for the proliferation of axillary buds in avocado, and additionally it was also used as an opportunity to develop an understanding of how avocado explants respond to the *in vitro* environment, as this would allow for the system to be improved so that healthy and vigorously growing material can be generated for cryopreservation.

1.1.5 Rationale

As the planting and trade of avocados becomes more widespread, and with an increase in the awareness of the health benefits of avocado, avocado may become an important food security fruit crop (Schaffer *et al.*, 2013b). Avocado fruits provide a rich source of oils, vitamins, proteins, water and minerals, and consequently it has the potential to play an important role in solving the problem of hunger and malnutrition in the future (Schaffer *et al.*, 2013b). Because avocado produces a recalcitrant seed, the germplasm of avocado cannot be stored using common seed storage practices. *In vitro* tissue culture has therefore been considered as an alternative method of propagating avocados. The long term conservation of avocado genetic material could thus be achieved through the cryopreservation of tissue culture-generated axillary buds.

1.1.6 Aims and objectives of the study

The first aim of this study was to establish an *in vitro* system for the proliferation of axillary buds in avocado (*P. americana*) cv. ‘Edranol’, for the purpose of cryopreservation. The second aim was to develop an understanding of how the avocado explants respond to the *in vitro* environment.

2 CHAPTER TWO - Initial Tissue Culture Experiments

2.1 INTRODUCTION

2.1.1 The micropropagation of avocado (*P. americana*)

As with many other woody species, the tissue culture of avocado has not been without difficulty (Barceló-Muñoz and Pliego-Alfaro, 2012; George and Deberg, 2008). The main limits to the successful vegetative propagation of many woody species include the browning of tissues and media, tissue vitrification and apical necrosis, all of which may be due to the production and accumulation of secondary metabolites (such as phenolic compounds) and other substances that inhibit growth (Gogoi and Borua, 2014; George and Deberg, 2008). Therefore, pretreatments may be required to successfully introduce woody species into the *in vitro* environment (George and Deberg, 2008; Schroeder, 1980).

The mass multiplication of avocado vegetative material through tissue culture has had limited success (Bandaralage *et al.*, 2015; Barceló-Muñoz and Pliego-Alfaro, 2012; Ahmed *et al.*, 2001). Thus far, avocado tissues that have been cultured *in vitro* include leaf tissues, embryonic axis of seeds and vegetative shoots, and the majority of this work has been conducted using the 'Hass' and 'Fuerte' cultivars (Rohim *et al.*, 2013; Zulfiqar *et al.*, 2009; Fuentes *et al.*, 2004; Barceló-Muñoz *et al.*, 1999).

The *in vitro* propagation of avocado is discussed with a focus on mother plant selection and preparation (stage 0), the establishment of an aseptic culture (stage 1) and the multiplication of suitable propagules (stage 2). Thereafter, some physiological responses which were observed for avocado explants to the *in vitro* environment are considered. These responses include tissue browning, hyperhydricity and callus formation.

2.1.1.1 Stage 0. Mother plant selection and preparation

2.1.1.1.1 Explant source / starting material

The physiological stage of development of the mother plants may have an effect on how the material responds in culture (George and Deberg, 2008; Razdan, 2003; Bhojwani and Razdan, 1996). For the *in vitro* propagation of avocado, starting material was reported to be obtained from seed embryos and vegetative tissue (e.g. apical buds and axillary buds) from established plants. Although *in vitro* growth has been achieved by using mature material, Ahmed *et al.* (1997) stated that there was difficulty in developing a reproducible system for

the tissue culture of avocado from mature tissues. This is because in culture, mature material has been associated with slow growth rates and apical necrosis (Barceló-Muñoz and Pliego-Alfaro, 2012; Zulfiqar *et al.*, 2009; Minocha and Jain, 2000; Cooper, 1987; Schall, 1987; Harty, 1985). Thus, juvenile material is generally favoured for avocado tissue culture (Barceló-Muñoz and Pliego-Alfaro, 2012; Barceló-Muñoz *et al.*, 1999). Juvenile material has been described both as growth from juvenile trees and new growth from mature trees. With avocado research, the specific trees of interest (e.g. bud wood mother plants) are often old and have been growing in plantations for many years. In such instances, grafting has been used to rejuvenate the material to achieve vigorous growth *in vitro* (Nhut *et al.*, 2008; Barceló-Muñoz *et al.*, 1999; Pierik, 1990; Pliego-Alfaro and Murashige, 1987).

2.1.1.1.2 Primary explant type

Vegetative propagation through tissue culture can be achieved by using a variety of plant organs, including shoots tips, which contain an apical meristem and leaf primordia, and axillary or lateral buds (Barceló-Muñoz and Pliego-Alfaro, 2012; George, 2008; Razdan, 2003; Bhojwani and Razdan, 1996). Axillary bud culture involves the growth of multiple shoots from axillary buds and can be achieved through the application of cytokinins (Barceló-Muñoz and Pliego-Alfaro, 2012; George, 2008; Razdan, 2003; Bhojwani and Razdan, 1996). Axillary bud culture is a method of direct organogenesis, meaning that new shoots can develop directly from meristems, and thus does not require an intermediate callus phase (Ngezahayo *et al.*, 2014; Iliev *et al.*, 2010; Chawla, 2002). In this way, axillary bud culture is considered to be a relatively simple cloning method of propagation compared with other more complex methods (Barceló-Muñoz and Pliego-Alfaro, 2012; Razdan, 2003; Chawla, 2002; Grout, 1999; Bhojwani and Razdan, 1996). It is for this reason that axillary buds were chosen as the primary explants in this study.

2.1.1.2 Stage 1. Establishing an aseptic culture

The successful micropropagation of plant material requires the establishment of an aseptic culture (Zulfiqar *et al.*, 2009; George and Deberg, 2008; Chawla, 2002; Barrera-Guerra *et al.*, 2001; Barringer *et al.*, 1996). Contamination of *in vitro* plantlets by microorganisms may lead to decreased *in vitro* productivity and loss of explants. While microorganisms are present in the natural environment, in culture certain treatments/ conditions may favour the proliferation of microorganisms more than other treatments (Zulfiqar *et al.*, 2009; George and Deberg, 2008; Chawla, 2002; Barrera-Guerra *et al.*, 2001; Barringer *et al.*, 1996). To successfully

eradicate contamination *in vitro*, decontamination protocols need to be optimised (Kane, 2016). This is because exposure to decontaminants (e.g. sodium hypochlorite) in high concentrations, or for extended periods may bring about oxidation of explants, and this is of particular importance for plant material that naturally oxidises easily, such as that of avocado (Zulfiqar *et al.*, 2009; George and Deberg, 2008; Chawla, 2002; Barrera-Guerra *et al.*, 2001; Barringer *et al.*, 1996). Furthermore, in certain varieties of avocado, such as 'Ouro Verde', antibiotics (Nalidixic acid, Chloramphenicol and Streptomycin) have been found to be toxic (Biasi, 1995). Thus, the aim of surface sterilisation is to eliminate contamination, while preserving the health and vigour of the explant.

2.1.1.3 Stage 2. Multiplication of suitable propagules

2.1.1.3.1 Basal medium

In terms of media composition for avocado vegetative propagation, some authors have used basal media with lower salt concentrations such as Gamborg's B5 medium (Rohim *et al.*, 2013; Gamborg *et al.*, 1968) and Woody Plant Medium (Castro *et al.*, 1995; McCown and Lloyd, 1981). Even though it was found to be toxic for some varieties of avocado (Harty, 1985), most authors confirm that Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), either in half or full strength, is a suitable source of nitrogen, vitamins and the other essential macro- and micro- nutrients for avocado micropropagation (Table 2). The reason for MS being toxic for woody species may be due to it being more adapted to species with herbaceous growth (Schroeder, 1980), such as tobacco for which it was originally developed (Murashige and Skoog, 1962). It has been stated that the high ionic strength of basal medium, such as MS, may inhibit growth in woody species (McCown and Sellmer, 1987). Nitrogen requirements differ across species and for the growth of woody trees, less nitrogen is needed than by crop species (Pallardy, 2010).

Some authors have used an initiation medium to proliferate shoots, the axillary buds of which were then cultured on a multiplication medium (Table 2). Initiation media that were used for avocado varieties mostly comprised of MS supplemented with BAP in different concentrations (Table 2). Other authors, however, have used a single medium for both the initiation and multiplication of *in vitro* axillary bud cultures (Table 2).

2.1.1.3.2 Plant growth regulators

The auxins and cytokinins have the biggest impact on growth and development in plants (Razdan, 2003). Optimum growth can only be achieved when the correct balance of plant growth regulators is present (Machakova *et al.*, 2008; Razdan, 2003; Shimizu-Sato and Mori, 2001; Bhojwani and Razdan, 1996). Auxins are found at high concentrations in apical meristems where they are synthesised, but may also be synthesised in leaves (Machakova *et al.*, 2008; Razdan, 2003; Bhojwani and Razdan, 1996). Apical dominance is controlled by auxins (Müller and Leyser, 2011; Machakova *et al.*, 2008). The removal of apical dominance, allowing for the proliferation of multiple shoots from isolated axillary buds *in vitro* occurs at a high cytokinin to auxin ratio (Müller and Leyser, 2011; Van Staden *et al.*, 2008; Razdan, 2003; Shimizu-Sato and Mori, 2001; Ahmed *et al.*, 1997). However, even with the addition of cytokinin, certain species such as avocado display strong apical dominance (Barceló-Muñoz and Pliego-Alfaro, 2012; Mohamed-Yasseen, 1993; Castro *et al.*, 1995; Nel and Kotzé, 1984).

Cytokinins are involved in growth and development through cell division (Machakova *et al.*, 2008; Razdan, 2003; Shimizu-Sato and Mori, 2001; Bhojwani and Razdan, 1996). The addition of cytokinins alone to the culture medium was found to be sufficient for inducing axillary bud growth from *in vitro* avocado cultures (Table 2). This suggested that the *in vitro* plantlets produce sufficient levels of (endogenous) auxins, and inadequate levels of cytokinins, and thus the cultures needed to be supplemented with exogenous cytokinins (Bandaralage *et al.*, 2015; Barceló-Muñoz and Pliego-Alfaro, 2012; Van Staden *et al.*, 2008; Minocha and Jain, 2000). The cytokinin 6-Benzylaminopurine (BAP), sometimes referred to as Benzyladenine (BA) (da Silva, 2012; Van Staden *et al.*, 2008) was reported to be preferred for axillary bud growth in avocado, in concentrations ranging from 0.5mg/l to 3mg/l (Table 2). BAP in concentrations above 4mg/l has been shown to cause vitrification of *in vitro* avocado tissues (Zulfiqar *et al.*, 2009; Van Staden *et al.*, 2008; Fuentes *et al.*, 2004).

2.1.1.3.3 Media texture

Double-phase and semi-solid media have been used for avocado micropropagation (Zulfiqar *et al.*, 2009; Barceló-Muñoz *et al.*, 1999; Castro *et al.*, 1995; Pliego-Alfaro *et al.*, 1987). Double-phase media have been used to reduce apical necrosis, which has been attributed to deficiencies in nutrients (calcium, potassium, boron) and cytokinins in apical shoot tips (Bairu *et al.*, 2009; Kataeva *et al.*, 1991). Tissues cultured on double-phase media have, however, been associated with hyperhydricity and succulent growth (de la Viña *et al.*, 2001;

Barceló-Muñoz *et al.*, 1999; Pliego-Alfaro *et al.*, 1987). It is because of this that most authors have reported the use of semi-solid media (Table 2). For solidification, agar has been more commonly used (Table 2), although some researchers have preferred to use Gelrite (Sánchez-Romero *et al.*, 2007; Castro *et al.*, 1995). Gelrite is generally preferred for solidification as it contains fewer impurities and is more economical than agar (George *et al.*, 2008). In addition to this, Gelrite sets as a clear gel and this makes visualising contamination in the *in vitro* cultures easier (George *et al.*, 2008).

Table 2. Summary of media and plant growth regulators that have been used for the *in vitro* propagation of avocado

Starting material	Variety	Surface decontamination	Medium	Plant growth regulators	Reference
Axillary buds	'Velvick'	Rinsed under running tap water (45 min.) 70 % (v/v) ethanol (3 min.) Rinsed 3 times with sterile distilled water (SDW) 3% (v/v) bleach solution and 3 drops of Tween 20 (3 min.) Rinsed several times with SDW	½ strength WPM (initiation) 20 g/l sucrose 2.5 g/l full strength WPM (regeneration) 2 mg/l $AgNO_3$ 20 g/l sucrose 2.5 g/l phytagel	2 mg/l Meta-topolin 0.1 mg/l GA_3	Bandaralage *et al.*, 2015
Zygotic embryos	'Reed' 'Hass' 'Duke 7' 'A10'	(Pliego-Alfaro and Murashige, 1987)	Induction (Litz and Witjaksono, 1999)		Encina *et al.*, 2014
Zygotic embryos	'Hass'	25% Clorox 5.25% (v/v) sodium hypochlorite with Tween 20 (1 drop/100 ml) (20 min.) 3 rinses in SDW	Full strength MS and B5 7g/l Bactoagarv 30 g/l sucrose	20 mg/l kinetin or 1mg/l BAP 0.2 mg/l IBA	Rohim *et al.*, 2013
Stem pieces	'Hass'	70% (v/v) ethanol 10% (v/v) Domestos solution (20 min.) 3 rinses in SDW	½ MS or B5 or WPM	1.5 mg/l BAP	Taah *et al.*,2009
Axillary buds	'Fuerte'	running tap water (half an hour) 1% sodium hypochlorite (NaOCl) solution with continuous agitation (10 min) 3 rinses (5 min. each) with SDW	¾ strength MS 30 g/l sucrose 6.5 g/l agar	1 mg/l BAP 1.5 mg/l IBA	Zulfiqar *et al.*, 2009
Juvenile sprouts Mature terminal shoots		1% w/v washing detergent powder (Viso, Viet Nam) (20 min) washed with running tap water (1 h) 70% ethanol (30 sec.) rinsed twice in SDW 1% HgCl2 with 2-3 drops of Tween 80 (7 min) 3 rinses in SDW	MS medium 3% (w/v) sucrose 8% (w/v) agar 2 % peptone		Nhut *et al.*, 2008

Embryos	West Indian and West Indian x Guatemalan avocados	-	B5 major Salts MS minor salts 100 mg/l *myo*-inositol 3g/l sucrose 8g/l TC agar	4.14 µM picloram 4 mg/l thiamine HCl	Litz *et al.*, 2007
Zygotic embryos	'Hass'	0.5% (v/v) sodium hypochlorite solution with1 drop/100 ml Tween 20 (10 min) 3 rinses in SDW	½ strength MS 1.7 g/l Gelrite	0.5 mg/l BAP 1 mg/l GA_3	Sánchez-Romero *et al.*, 2007
Shoots	'Hass' 'Booth-7' 'Lula' 'Lima Late' 'Waldin'	-	MS medium without NH_4NO_3 30 g/l sucrose	1 ml/l BAP	Raharjo and Litz, 2005
Zygotic embryos	'Duke' 'Hass' 'Suardía Estación' 'Catalina' 'Jaruco No. 1'	Seeds dipped into 90 % (v/v) ethanol and flamed	½ strength MS 30 g/l sucrose 100 mg/l *i*-inositol	0.5 mg/l BAP 0.5 mg/l GA_3	Fuentes *et al.*, 2004
Zygotic embryos	'Anaheim'	-	½ strength MS minerals, vitamins and sucrose	1 mg/l BAP	Perán-Quesada *et al.*, 2004
In vitro shoots	'Gvaram 13'	-	MS with the N_{45} K macroelement formulation (Margara, 1984) 6g/l A1296 agar	1 ml/l BAP	Premkumar *et al.*, 2002
Mature embryos	'Hass'	washed in running tap water (10 min.) surface sterilised by soaking in either 37.5 % (fruit only) or 25 % (seeds) commercial bleach (4 % w/v available chlorine) (20 min.) rinsed 3 times is SDW	MS medium 400mgl/1 casein hydrolysate 0.1% (w/v) PVP 3% (w/v) sucrose 0.7% agar	1 mg/l BAP 0.1 mg/l IBA 0.1 mg/l /GA_3	Ahmed *et al.*, 2001
Shoots	Mexican	20% sodium hypochlorite and 0.1% Tween 20 (30 min)	MS	80 mg/l adenine	Barrera-Guerra *et al.*, 2001

	race	2% Benlate for (30 min) 20% sodium hypochlorite (10 min) 4 rinses in SDW		sulphate 2 mg/l BAP	
Axillary buds	'RR-86'	0.5% sodium hypoclorite with a few drops of Tween (20 -10 min.) rinsed with water	½ strength MS 6 g/l agar (initiation) 1.7 g agar (proliferation medium)	0.65 mg/l BAP	de la Viña *et al.*, 2001
Apical shoots	'Hass'	0.5% sodium hypoclorite with a few drops of Tween (20-10 min.)	½ strength MS 6 g/l agar (solid phase)	0.3 mg/l BAP (initiation) Agitated liquid- 0.3 mg/l BAP followed by double phase medium: 0.65 mg/l BAP- solid 0.1 mg/l BAP- liquid	Barceló-Muñoz *et al.*, 1999
Zygotic embryo	Various varieties	10–20%commercial bleach containing 10–20 drops/l Tween 20 (10–20 min)	B5 major salts MS minor salts 0.4 mg/l thiamine HCl, 100 mg/l *myo*-inositol 30 g/l sucrose 8 g/l TC agar	0.41 µM picloram (induction)	Litz and Witjaksono, 1999
In vitro Shoot cultures	'Gvaram 13'	-	Modified MS with 67% KNO_3 and 33% NH_4NO_3 1 mg/l thiamine HCI 100 mg/l *myo*-inositol 30 g/l sucrose 8 g/l Tissue Culture Agar	1 mg/l BAP	Schaffer *et al.*, 1999
Immature embryos Mature embryos	'Hass' 'Suardía Station' 'Catalina Jaruco' 'No. 1'	Fruits- detergent and running water and immersed in 96% ethanol to be flamed in the flow chamber	½ MS Dixon and Fuller medium (Dixon and Fuller, 1976)	0.5 mg/l BAP 0.5 mg/l GA_3	Rodríguez *et al.*, 1997
Embryonic axes	'Catalonia' 'Dade' 'Maxima' 'Tower 2'	Seeds-1% detergent (Alconox) (10 min.) 0.8% (vol/vol) sodium hypochlorite (20 min.) 3 rinses in SDW Embryonic axes –0.1% (vol/vol) sodium hypochlorite (10 min.)	MS 8 g/l agar 30 g/l sucrose	3 mg/l BAP 1 mg/l NAA 1 mg/l BAP (shoot proliferation) 0.1mg/l NAA	Barringer *et al.*, 1996

		3 rinses in SDW			
Shoot tips Axillary buds	'Duke 7'	5 g/l Benlate (Benomil) (30 min) 70 % ethanol (2 min.) 3.5 % sodium hypochlorite (15 min.) Rinsed with SDW	Modified MS with 40 ml/l FeNaEDTA instead of $FeSO_4$-H_2O and Na_2EDTA 2 g/l Gelrite	1 mg/l BAP 0.1 mg/l IBA	Wessels, 1996
Axillary buds	'Lula' 'Velvick'	95% ethanol (5 sec.) 0.5% sodium hypochlorite plus 0.1 ml/l Tween (20-20 min.) five rinses with antioxidant solution	WPM 0.4 mg/l thiamine 500 mg/l casein 80 mg/l glutamine 100 mg/l *myo*-inositol 1 g/l PVP 60 mg/l ascorbic acid 30g/l sucrose 0.2% agar 0.2% Gelrite	Lula 0.5 mg/l CPPU Velvick 0.1 mg/l CPPU	Castro *et al.*, 1995
Nodal explants	'Ouro Verde'	70% ethanol (1 min.) 1% sodium hypochlorite (10 mins)	MS 7g/l agar	1 mg/l BAP 10 mg/l IBA	Biasi, 1995
Nodal explants	'Ouro Verde'	70% ethanol (1 min.) 1% sodium hypochlorite (10 mins)	½ MS 30g/l sucrose 7g/l agar	3 mg/l BAP	Biasi *et al.*, 1994
Shoot tips	'Hass' 'Fuerte' 'Topa-Topa' 'Duke'	0.5% sodium hypochlorite (10 mins)	MS 30 mg/l sucrose 100 mg/l *i*-inositol 0.4 mg/l thiamine HCl 8 mg/l agar	0,65 mg/l BAP-solid phase 0.1 mg/l BAP-liquid phase	Zirari and Lionakis, 1994
Embryonic axis	'Dade' 'Maxima' 'Cataloina' 'Tower 2' 'Waldin' 'Choquette'	Seed coats removed and seeds washed with detergent 20% chlorox for (20 min) embryonic axes: 5% chlorox (15 min)	MS 100 mg/l *myo*-inositol 30 g/l sucrose 8 g/l agar	3 mg/l BAP 2 mg/l Thidiazuron 0.1 mg/l NAA 2 mg/l IBA	Mohamed-Yasseen, 1993
Etiolated buds	'Colin V-33' West Indian strain	disinfected, rinsed, and soaked in cloralex with 10% of active ingredient (10 min.)	MS 100 mg/1 *myo*-inositol 3 % sucrose	2 mg/l BAP 2 mg/l GA_3	Vega, 1989

		rinsed again	4% thiamine 2 g/l Gelrite		
Embryos	-	Seeds – 80% ethanol Embryo- 15% Clorox solution	MS (modified) 2mg/l activated charcoal	1 mg/l NAA or IAA 1 mg/l Kinetin, BAP or 2iP	Chin *et al.*, 1988
Zygotic embryos	'Hass'	0.5 % sodium hypochlorite with 0.1% Tween 20 (10 min.) 3 rinses in SDW	MS 30 mg/1 sucrose 0.4 mg/l thiamine.HC1 100 mg/1 *i*-inositol 8% agar	0.01 mg/l picloram	Pliego-Alfaro and Murashige, 1988
Axillary buds	'Duke 7'	95% ethanol (2-3 sec.) 0.5% sodium hypochlorite with 0.05% Multifilm wetting agent X77 (30 min.) 3 rinses in SDW	WPM with minerals and vitamins 30 g/l sucrose 7 g/l agar	1 mg/l BAP (for initiation) 0.3 mg/l BAP (multiplication) 0.1 mg/l indole butyric acid	Cooper, 1987
Shoots (lateral buds)	'IV-8' rootstock	0.5 % sodium hypochlorite solution (10 min.)	½ MS 30 mg/l sucrose 100 mg/l *i*-inositol 0.4 mg/l thiamine HCl 8 mg/l agar	0.65 mg/l BAP-solid phase 0,1 mg/l BAP-liquid phase	Pliego-Alfaro *et al.*, 1987
	'GA-13' rootstock		Macro-elements $N_{45}K$ (Margara, 1984) MS Microelements 30 mg/l sucrose 100 mg/l *i*-inositol 0.4 mg/l thiamine HCl 8 mg/l agar	1 mg/l BAP	
Shoots	'Duke 7'	-	MS macro- and microelements Morel vitamin mixture (Morel and Wetmore, 1951) 25 mg/l FeEDTA 170 mg/l NaH2PO4 100 mg/l *myo*-inositol 30 g/l sucrose	5-10 mg/l BAP	Schall, 1987

Shoot tips	'Duke 7'	1% sodium hypochlorite with Tween 20- (15 min.) rinsed in SDW	Dixon and Fuller medium (Dixon and Fuller, 1976)	40 mg/l L-glutamine 40 mg/l L-arginine 55.7 mg/l FeSO4.7H2O 10 mg/l of kinetin	Harty, 1985
Stem pieces	'Antillana'	70% (v/v) ethanol (1 min.) 4% (v/v) calcium hypochloride (20 min.) rinsed in SDW	MS	0.3 mg/l IBA 0.1 mg/l of Kinetin	Gonzalez-Rosas and Salazar-García, 1984
Axillary buds	'Lula' 'Waldin'	benomyl (1.46 g/l) and captan (2.3 g/l) with constant agitation (15-30 min.) storing overnight at 5°C soaking in filtered 9% CaOCl and 0.1% Tween 20 (30-60 min.) 3 rinses in SDW	Anderson's mineral salts and vitamins 1g/l caScin hydrolysate 30 g/l sucrose 6 g/l Bactoagar	0.2 mg/l BAP 0.2 mg/l IBA 1 mg/l GA_3	Young, 1983
Shoot tips	'Duke 7' 'Edranol'	-	Macro-elements: ½ MS Microelements: Full MS Modified with 25mg/l FeNaEDTA 30mg/l sucrose 170mg/l NaH_2PO_4 $2H_2O$ 80mg/l adenine sulphate 25mg/l ascorbic acid 100mg/l *myo*-inositol 2mg/l glycine 1mg/l pyridoxine HCl 1mg/l thiamine HCl 1mg/l nicotinic acid 1mg/l pantothenic acid (Ca-salt)	2 mg/l BAP 1 mg/l GA_3	Nel *et al.*, 1983
Immature embryos Mature and hybrid embryos	'Fuerte' and hybrids	Seed -dipped in 90 % (v/v) ethanol and flamed to surface sterilize	½ MS (minerals, vitamins and sucrose)	0.5mg/l BAP	Skene and Barlass, 1983

Buds (axillary)		1% NaOCI (15 min.) rinsed in SDW	½ MS macro-elements MS micro-elements (modified) 1 mg/l thiamine HCl 1mg/l calcium pantothenate 100mg/l inositol 30g/l sucrose	2 mg/l BAP 0.5 mg/l GA_3	Nel and Kotzé, 1982
Stem apices	'Duke' 'Fuerte'	0.3% sodium hypochlorite (10 min.) 3 rinses in SDW	Millers medium (Miller, 1963) with agar	1 mg/l kinetin 1 mg/l NAA	Hendry and Van Staden, 1982

2.1.2 Some physiological responses of avocado explants to the *in vitro* environment

2.1.2.1 Browning

Amongst the factors limiting the successful *in vitro* propagation of avocado is tissue browning or oxidation, associated with the production of ethylene (Schall, 1987), and necrosis (Rohim *et al.*, 2013; Zulfiqar *et al.*, 2009; Fuentes *et al.*, 2004; Castro *et al.*, 1995; Pliego-Alfaro and Murashige, 1988). Strategies which have been used to reduce browning *in vitro* include the use of double-phase media, the addition of antioxidants, such as polyvinylpyrrolidone (PVP) (Ahmed *et al.*, 2001; Dalsaso and Guevara, 1989), or charcoal to the culture medium (Pliego-Alfaro and Murashige, 1988, 1987). Additionally, some authors have used low temperature pretreatments to control tissue browning (Barrera-Guerra *et al.*, 2001; Barceló-Muñoz *et al.*, 1999; Castro *et al.*, 1995). Most authors, however, have agreed that initial culture in the dark alone is sufficient for reducing browning and necrosis in avocado *in vitro* tissues (Rohim *et al.*, 2013; Barringer *et al.*, 1996; Castro *et al.*, 1995; Mohamed-Yasseen, 1993). The excision of explants from mother plants at *in vitro* establishment and at subculture leads to the oxidation of phenolic compounds (George, 2008). In the presence of light, the activity of the enzymes which are associated with the oxidation of these phenolic compounds increase, which in turn leads to the browning of tissues (Thomas, 2008). Thus, culturing explants in the dark for 7 to 10 days after both isolation and subculture reduces the activity of these enzymes, which then reduces the tissue browning of avocado tissues (Rohim *et al.*, 2013; Barringer *et al.*, 1996; Castro *et al.*, 1995; Mohamed-Yasseen, 1993). Other authors, however, still observed low survival rates, browning and necrosis when using antioxidants, charcoal, low temperature pretreatment and darkness (Barrera-Guerra *et al.*, 2001; Barceló-Muñoz *et al.*, 1999; Castro *et al.*, 1995).

2.1.2.2 Hyperhydricity

Hyperhydricity has been identified as another limitation for the successful *in vitro* propagation of avocado (Bandaralage *et al.*, 2015; Barceló-Muñoz and Pliego-Alfaro, 2012; Litz *et al.*, 2005; de la Viña *et al.*, 2001; Barceló-Muñoz *et al.*, 1999; Zirari and Lionakis, 1994). Hyperhydricity is caused mainly by the high relative humidity and lack of ventilation in the *in vitro* environment (Chen *et al.*, 2006; García-Gonzáles *et al.*, 2010; Apóstolo and Llorente, 2000; Ziv, 1991; McClelland and Smith, 1990). Hyperhydricity can also be caused by certain plant growth regulators and gelling agents (Apóstolo and Llorente, 2000; Debergh

et al., 1992; Ziv, 1991). In their study, de la Viña *et al.* (2001) determined that characteristics of hyperhydricity in avocado tissues *in vitro* included an increase in tissue water content, larger and deformed stomata and an increase in epicuticular wax deposits. In other species, higher levels of irradiation were beneficial at reducing hyperhydricity *in vitro* (de la Viña *et al.*, 2001). However, in avocado cv. 'Topa Topa' it was observed that hyperhydricity was not successfully reduced with this treatment (de la Viña *et al.*, 2001).

2.1.2.3 Callus

Callus is a form of unorganised growth, which can be induced at a high auxin to cytokinin ratio and can occur as a wounding response (Ikeuchi *et al.*, 2013; George and Deberg, 2008; Razdan, 2003; Dodds and Roberts, 1985). An *in vitro* explant contains many types of tissues which are composed of different cell types; the dedifferentiation of these cells may give rise to callus with different textures and colours (Ikeuchi *et al.*, 2013; George and Deberg, 2008; Razdan, 2003; Dodds and Roberts, 1985). Different colours of callus come about as a result of different pigments in the tissues (Ikeuchi *et al.*, 2013; George and Deberg, 2008; Razdan, 2003; Dodds and Roberts, 1985).

2.1.3 Aims and objectives

The aim was to develop a system for the establishment and multiplication of *in vitro* avocado axillary buds. The objective of this aim was to select the most appropriate medium for the proliferation of axillary buds from avocado plantlets, based on physiological measurements.

2.2 MATERIALS AND METHODS

2.2.1 Experimental design and protocols

2.2.1.1 Stage 0. Mother plant establishment and preparation

Six-month-old 'Edranol' avocado seedlings were sourced from Rietvlei Nursery, Tzaneen. The plants were maintained with a 16-hour photoperiod at 26°C and 50% relative humidity in the Phytotron at the University of the Witwatersrand, Johannesburg (Figure 1). The plants were potted in a soil mix of perlite, compost, commercial potting soil (Culterra brand) in 20L plastic bags. Watering was carried out by hand and each plant was given 250ml water per week in summer and 250ml every second week in winter. The plants were treated with fertilisers, fungicides and insecticides according to the regime outlined in Table 3. Fungicides

(Previcur and Bravo 720®) and fertilisers were used alternatively once a month. The systemic fungicide Ridomil Gold was applied every 6 weeks to control root rot.

Figure 1. The 'Edranol' avocado seedlings were maintained in a Phytotron at the University of the Witwatersrand, Johannesburg.

Table 3. The regime of fertilisers, fungicides and insecticide used to treat the avocado mother plants prior to experimental use

Chemical type	Chemical Name	Brand	Active ingredient	Concentration used	Applied
Fertiliser	LAN	Efekto	Limestone Ammonium Nitrate	5g/20 litre bag	Every second month
	3:1:5	Efekto	-	5g/20 litre bag	
	Bone meal	Efekto	Bone phosphate Contains: N: P: Ca	5g/20 litre bag	
Fungicide	Previcur	Bayer	Propamocarb	1ml/l	Every second month
	Bravo 720®	Efekto	Chlorothalonil	2ml/l	
Insecticide	Malasol	Efekto	Mercaptothion	2.5ml/l	Once every two months

2.2.1.2 Stage 1. Establishment of an aseptic culture - Surface decontamination

Nodal cuttings were excised from the seedlings in the Phytotron. The explants were first rinsed under running tap water. Thereafter, the leaves were trimmed to allow for the explants to fit in a beaker (Figure 2). The explants were dipped into 70% ethanol for 30 seconds then rinsed again with running tap water. Under sterile conditions, the explants were surface decontaminated with 1% (v/v) sodium hypochlorite and Tween 20® (1 drop/ 100ml) for 10 minutes (Sánchez-Romero *et al.*, 2007; de la Viña *et al.*, 2001; Castro *et al.*, 1995). Thereafter the explants were rinsed three times with ultrapure (Millipore Milli Q®) autoclaved water, and then trimmed into 1-2cm segments, with each segment containing axillary buds (Figure 3). In some cases, individual axillary buds could not be separated without being damaged, thus each segment contained 1-3 axillary buds at *in vitro* establishment.

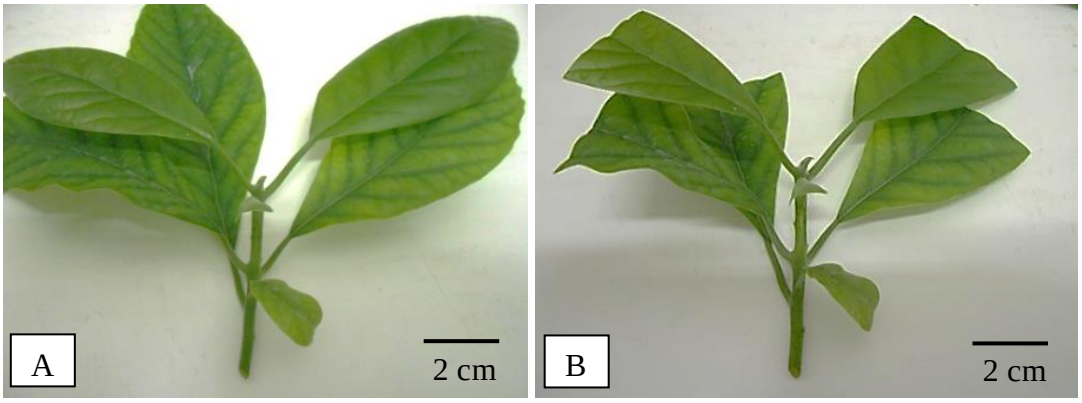


Figure 2. An avocado shoot before (A) and after (B) leaves were trimmed prior to surface decontamination.

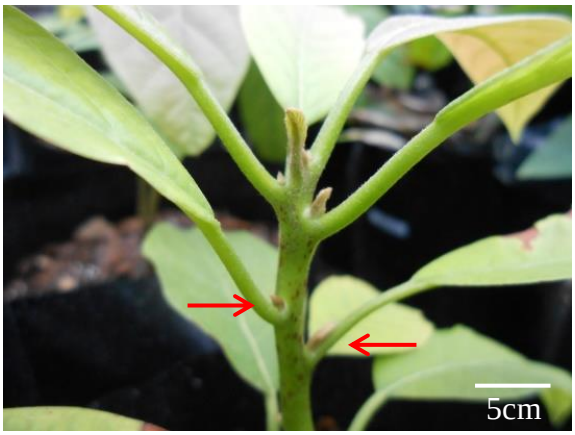


Figure 3. Axillary buds on an avocado mother plant.

2.2.1.3 Stage 2. In vitro establishment and multiplication

After surface decontamination, the explants were plated on 50ml semi-solid medium, in Magenta jars, 1-2 explants per jar (Figure 4). The tested media were MS with vitamins at full (4.48g/l) or half (2.24g/l) strength with BAP at either 0.5mg/l or 1mg/l. All media were supplemented with 30 g/l sucrose and solidified with 3g/l Gelrite, at pH 5.6 to 5.8. A drop of food colouring (Robertsons brand) was used to identify each of the media. The explants were initially cultured in the dark for 7-10 days at 25°C. Thereafter the explants were transferred to a 16-hour photoperiod.

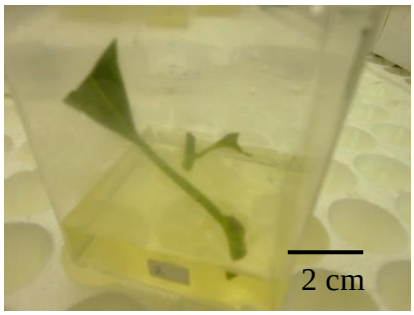


Figure 4. Axillary buds plated onto tissue culture medium in Magenta jars at *in vitro* establishment.

2.2.1.4 Physiological measurements

The explants were observed for 6 weeks after dark incubation and measurements of viability and vigour were taken. These included:

- the percentage (%) of contaminated explants
- the percentage (%) of explants that died
- the percentage (%) of hyperhydric explants
- the number of shoots developed
- the number of axillary buds developed
- Visual assessments of vigour included the extent of tissue browning (Table 4) and callus formation (Table 5).

Table 4. Scoring system that was used to visually assess tissue browning in *in vitro* avocado plantlets.

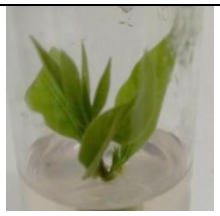
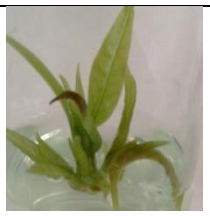
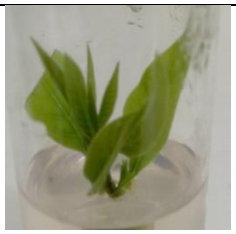
Score	**Criteria for score**	
0	No browning observed	
1	Browning on leaves, stem or axillary buds	
2	Browning on leaves and /or stem and /or axillary buds	
3	Increased browning on leaves and /or stem and /or axillary buds	
4	Widespread browning on explant with regions of pale green tissue	
5	Browning on entire explant, indicating explant death	

Table 5. Scoring system that was used to visually assess callus formation in *in vitro* avocado plantlets.

Score	**Criteria for score**	
0	No callus observed	
1	Callus on leaves, stem or axillary buds	
2	Callus on leaves and /or stem and /or axillary buds	
3	Increased callus formation on leaves and /or stem and /or axillary buds	
4	Widespread callus on explant	
5	Callus found on entire explant	N/A

2.2.1.5 Subculture

Six weeks after dark incubation the shoots which had developed from the explant nodal sections were cut into segments containing axillary buds (Figure 5). The explants were placed in individual test tubes containing 10ml of the media. After each subculture the explants were incubated in the dark for 7-10 days. At subculture the length (cm) of the shoot which had formed was measured.

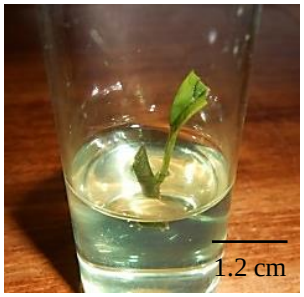


Figure 5. An axillary bud explant after subculture.

2.2.1.6 Sample numbers

The experiment comprised of 5 replicates, each with an uneven number of samples (between 5 and 7 samples per replicate). The total sample number for initial establishment experiments (Table 5) depended on the amount of material which could be harvested from avocado mother plants. Slow growth and development of the avocado mother plants meant that only a limited number of explants could be harvested for experimentation. For each of the subsequent subcultures, the sample numbers depended on the multiplication of the axillary buds on each of the media tested, after considering the loss of material due to contamination, death and hyperhydricity.

2.2.2 Data analysis

The data presented is per axillary bud explant. A Kruskal-Wallis ANOVA was used to determine if there was statistical significance between the four media tested. Percentages (contamination, hyperhydricity and death) were based on count data and were analysed using contingency table Chi-square tests. When counts were less than 5, a Fishers exact test was used (*p* values presented). Lower case alphabets represent significant differences according to post-hoc multiple comparison tests. All statistical tests were conducted at *0.05* significance level and were done with the program STATISTICA.

2.2.3 Imagery

All images were taken with a Nikon Coolpix S6300 HD 16.0 Megapixel camera.

2.3 RESULTS

The physiological responses of the explants are presented for the axillary bud explants cultured on the four tissue culture media tested, six weeks after *in vitro* establishment, the first, the second and the third subculture. Graphs were used to compare the responses of the

explants between the media tested for each of the variables, while tables were used to compare the responses between subcultures for each media.

Due to a combination of contamination (Figure 6), death (Figure 8) and hyperhydricity of explants (Figure 10) by the second subculture there were no viable explants from those cultured on 1MS + 0.5mg/l BAP, and by the third subculture this was also true for explants cultured on ½ MS + 0.5mg/l BAP (Table 5).

Table 5. The number of viable samples used in this study from *in vitro* establishment to the third subculture.

Medium	Total number of samples			
	Establishment	First subculture	Second subculture	Third subculture
½ MS + 0.5mg/l BAP	25	64	28	0
½ MS + 1 mg/l BAP	24	54	77	55
1 MS + 0.5mg/l BAP	26	24	0	0
1 MS + 1mg/l BAP	23	29	51	38

2.3.1 Stage 0. Mother plant establishment and preparation

Approximately a month after establishment the avocado mother plants began showing signs of stress which included leaf browning and curling. Additionally, leaf drop and branch die-back was observed. When the plants were uprooted their roots were black and underdeveloped, with few fine feeder roots. Uprooting the plants also exposed that the potting soil which was used had been waterlogged, even though irrigation was controlled. Overall, more than 50% of mother plants had died (data not shown) which limited the amount of plant material which could be harvested for experimentation.

2.3.2 Stage 1. Establishment of an aseptic culture

The contamination of explants was observed to be less than 30% for all the media tested, from establishment to the third subculture (Figure 6). During the first subculture, the highest percentage of contaminated explants (24.24%) was observed on 1MS + 0.5mg/l BAP, however this did not differ significantly from explants cultured on ½ MS + 0.5mg/l BAP (Figure 6.B). During the third subculture, a significantly higher percentage of contamination was observed in explants cultured on ½ MS + 0.5mg/l BAP (24.32%) compared with the other media tested (Figure 6.C). Between ½ MS + 1mg/l BAP and 1MS + 1mg/l BAP, there was no significant difference in the percentage contamination of explants observed from establishment to the second subculture (Figure 6.A-C).

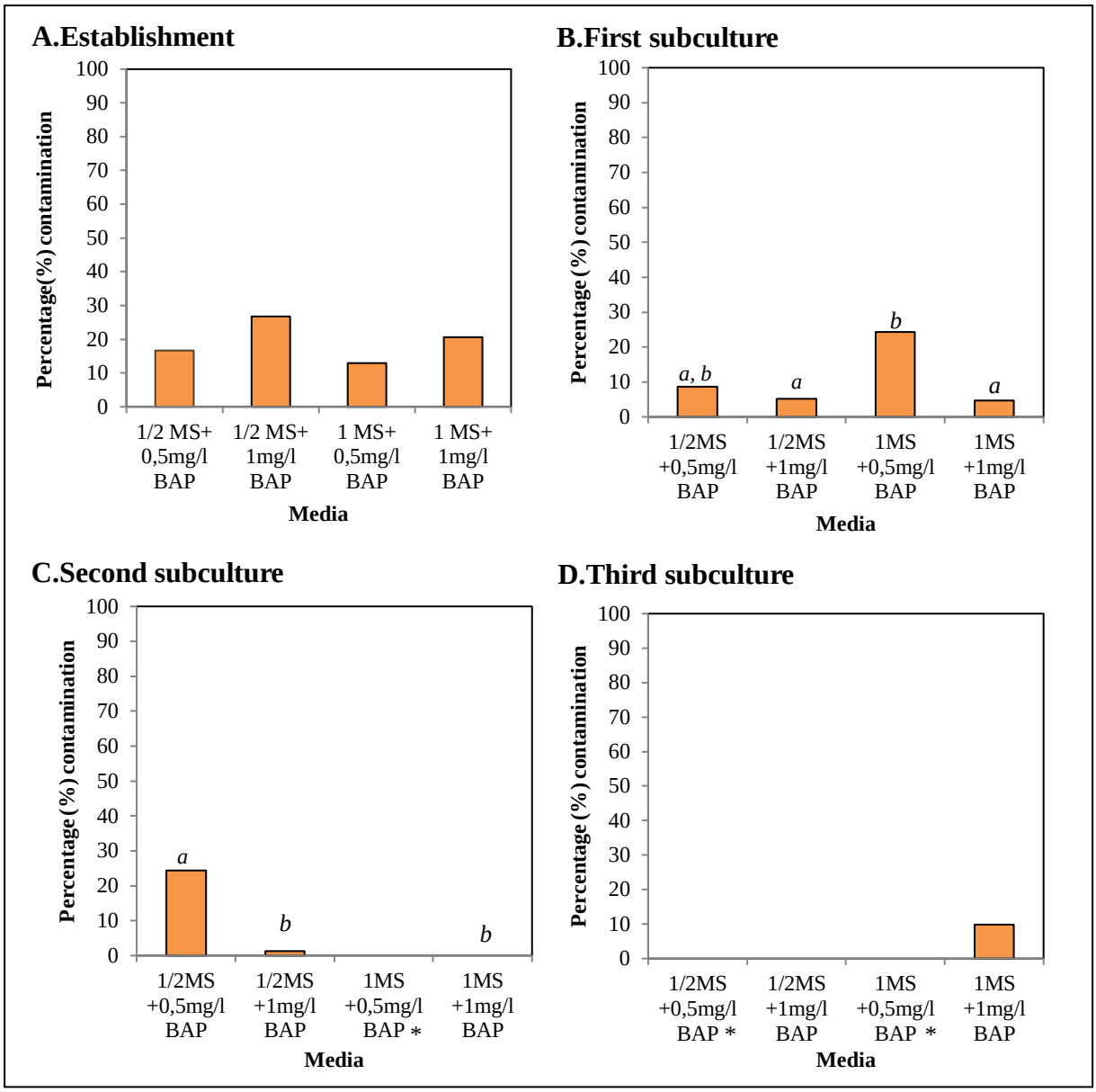


Figure 6. Percentage (%) contamination of *in vitro* avocado axillary bud explants cultured on four tissue culture media within 6 weeks of establishment (A), the first subculture (B), the second subculture(C) and the third subculture (D). There was no relationship between media and contamination at establishment (*p=0.06*). There was, however, a relationship between media and contamination at the first subculture (*p=0.013*), at the second subculture (*p<0.0001*) and at the third subculture (*p=0.01*). * No samples available.

For explants cultured on ½ MS + 0.5mg/l BAP contamination was numerically higher during the second subculture (24.32%) compared with establishment (16.6%) (Table 6). Similarly, for explants cultured on 1MS + 0.5mg/l BAP contamination was numerically higher during the first subculture (24.24%) than at establishment (18.87%) (Table 6). There was a significant decrease in the percentage contamination with explants cultured on ½ MS + 1mg/l BAP and 1MS + 1mg/l BAP from establishment to the third subculture (Table 6).

Table 6. Percentage (%) contamination of *in vitro* avocado axillary bud explants cultured on four tissue culture media within 6 weeks of establishment, the first subculture, the second subculture and the third subculture.

Medium	Percentage (%) contamination				
	Establishment	First subculture	Second subculture	Third subculture	*p* values
½ MS + 0.5mg/l BAP	16.66	8.57	24.32	-	*p=0.07*
½ MS + 1mg/l BAP	42.85^{a}	5.26^{b}	1.28^{b}	0^{b}	*p<0.0001*
1MS + 0.5mg/l BAP	18.87	24.24	-	-	*p=0.91*
1MS + 1mg/l BAP	37.14^{a}	4.76^{b}	0^{b}	9.75^{b}	*p<0.00001*

Lower case alphabets show significant differences amongst subcultures, according to statistical analysis conducted for each medium

2.3.3 Stage 2. *In vitro* establishment and multiplication

2.3.3.1 Tissue browning

From establishment to the third subculture, tissue browning scores ranged between 0.52 ± 0.14 and 2.77 ± 0.30 (Figure 7). There was no significant difference in the tissue browning scores of explants cultured on the four media at establishment (Figure 7.A). At second subculture a significantly higher tissue browning score (2.70 ± 0.27) was observed in explants cultured on ½ MS + 0.5mg/l BAP compared with the other media tested (Figure 7.C). There was no significant difference in the tissue browning scores observed for explants cultured on ½ MS + 1mg/l BAP compared with explants cultured on 1MS + 1mg/l BAP from establishment to the third subculture (Figure 7).

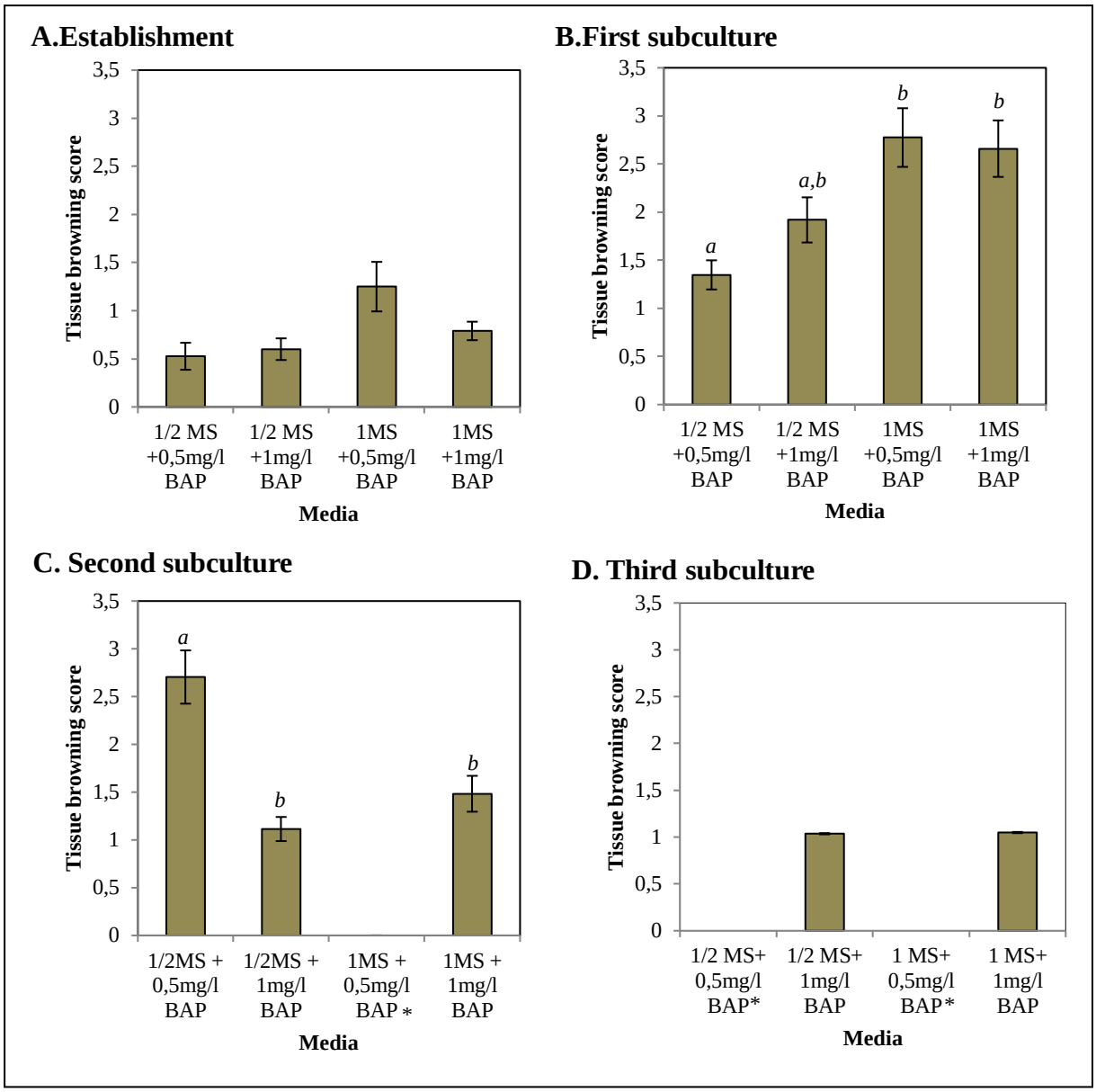


Figure 7. Tissue browning scores (mean ± SE) of *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment (A), the first subculture (B), the second subculture (C) and the third subculture (D). There was no significant difference in the tissue browning scores between the media at establishment (*H (81) =6.64, p=0.08*) and at the third subculture (*t (96) =-0.84, p=0.40*). There was, however, a significant difference in the tissue browning scores between the media at the first subculture (*H (206) =14.3, p=0.0025)* and at the second subculture (*H (180) =22.43, p<0.001*). * No samples available

Overall, there was an increase in the tissue browning scores for all the media tested from establishment to the first subculture (Table 7). There was a significant increase in the tissue browning score for explants cultured on ½ MS + 0.5mg/l BAP from establishment (0.52 ± 0.13) to the second subculture (2.70 ± 0.27) (Table 7).

Table 7. Tissue browning scores (mean ± SE) of *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment, the first subculture, the second subculture and the third subculture.

Medium	Tissue browning score				
	Establishment	First subculture	Second subculture	Third subculture	*p* values
½ MS + 0.5mg/l BAP	0.52± 0.13^{a}	1.34±0.15^{b}	2.70±0.27^{c}	-	*p <0.00001*
½ MS + 1mg/l BAP	0.6 ±0.11	1.91±0.23	1.11±0.12	1.03±0.08	*p >0.05*
1MS + 0.5mg/l BAP	1.2± 0.15	2.7± 0.28	-	-	*p=0.91*
1MS + 1mg/l BAP	0.79±0.09^{a}	2.65±0.29^{b}	1.48±1.86^{a}	1.04±0.09^{a}	*p=0.0004*

Lower case alphabets show significant differences amongst subcultures, according to statistical analysis conducted for each medium

2.3.3.2 Percentage death

There was no significant relationship between the percentage death of explants and media after establishment (Figure 8.A). After the second subculture a significantly higher percentage death (37.20%) was observed in explants cultured on ½ MS + 0.5mg/l BAP (Figure 8.C). There was no difference in the death of explants cultured on ½ MS + 1mg/l BAP and 1MS + 1mg/l BAP from establishment to the third subculture (Figure 8.D).

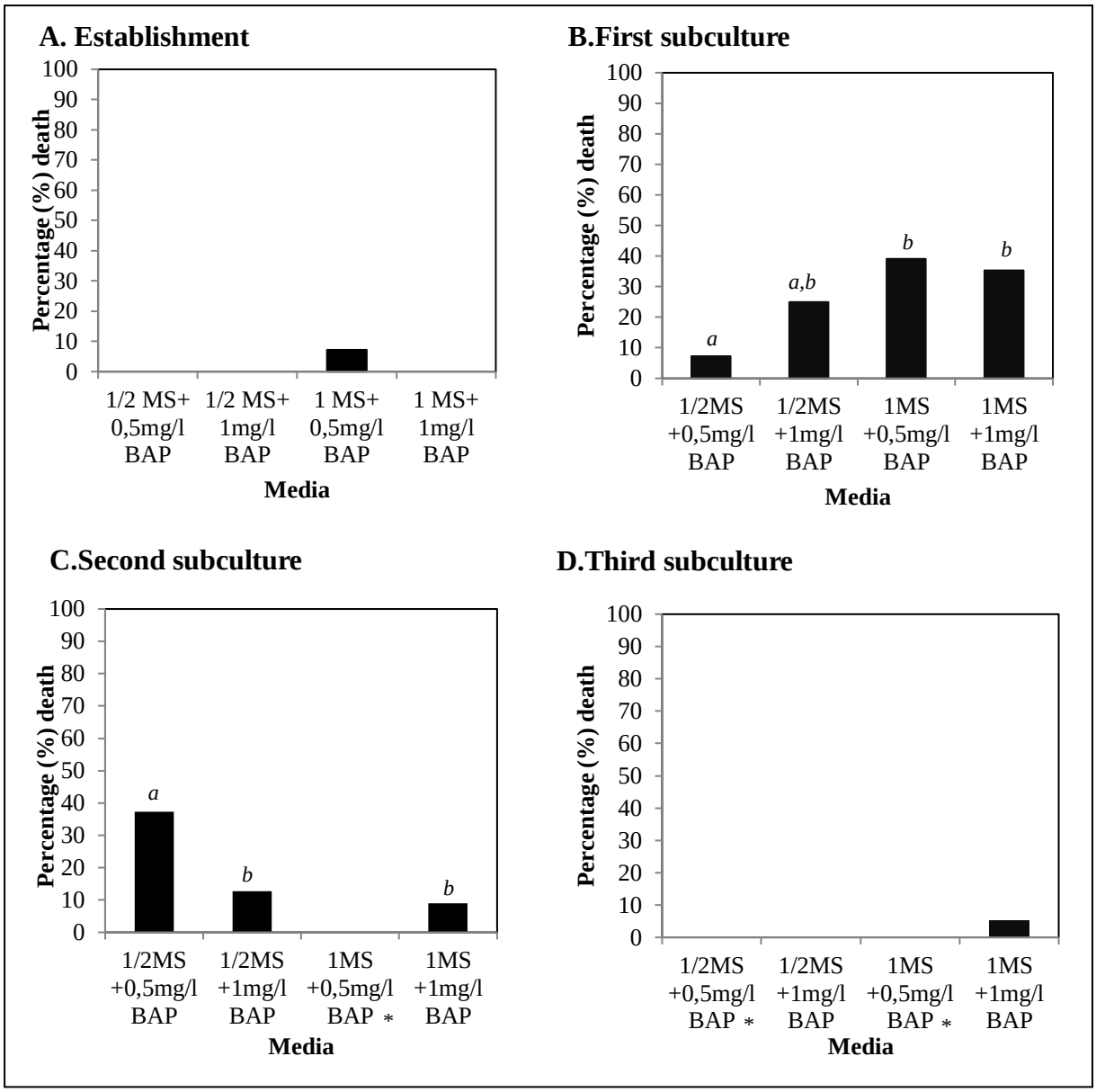


Figure 8.The percentage (%) death of *in vitro* avocado axillary bud explants cultured on four tissue culture media within 6 weeks of establishment (A), the first subculture (B), the second subculture (C) and the third subculture (D). There was no relationship observed between media and death at establishment (*p=0.16*) and at the third subculture (*p=0.08).* However, there was a significant relationship between media and death at the first subculture (*p<0.0001*) and at the second subculture (*p<0.001*)*).* * No samples available

For all the media tested there was an increase in the percentage death of explants from establishment to the first subculture (Table 8). For explants cultured on ½ MS + 0.5mg/l BAP the percentage death of explants increased significantly from establishment (0%) to the second subculture (37.2%) (Table 8). Similarly, for explants cultured on 1MS + 0.5mg/l BAP the percentage death of explants was significantly higher at the first subculture (39.02%) than at establishment (7.14%) (Table 8).

Table 8. The percentage (%) death of *in vitro* avocado axillary bud explants cultured on four tissue culture media within 6 weeks of establishment, the first subculture, the second subculture and the third subculture.

Medium	Percentage (%) death				
	Establishment	First subculture	Second subculture	Third subculture	*p* values
½ MS + 0.5mg/l BAP	0[a]	7.4[a]	37.20[b]	-	*p<0.00001*
½ MS + 1mg/l BAP	0	25	12.64	0	*p>0.05*
1MS + 0.5mg/l BAP	7.14[a]	39.02[b]	-	-	*p=0.004*
1MS + 1mg/l BAP	0	35.29	8.92	5.12	*p>0.05*

Lower case alphabets show significant differences amongst subcultures, according to statistical analysis conducted for each medium

2.3.3.3 Hyperhydricity

Hyperhydric explants appeared to have a transparent, watery appearance with leaves which were light green to yellow, thin and curled (Figure 9).

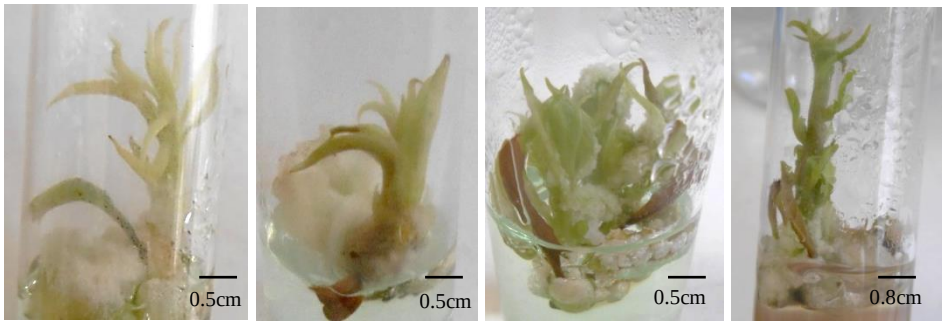


Figure 9. Symptoms of hyperhydricity in *in vitro* avocado axillary bud explants.

After establishment, there was no significant difference between the percentage of hyperhydric explants developed across the four media tested (Figure 10.A). After the second subculture a significantly higher percentage of hyperhydricity (42.85%) was observed in explants cultured on ½ MS + 0.5mg/l BAP (Figure 10.B). There was no significant difference between the percentage hyperhydricity observed in explants cultured on ½ MS + 1mg/l BAP compared with explants cultured on 1MS + 1mg/l BAP from establishment to the second subculture (Figure 10, A-C). After the third subculture, however, a significantly higher percentage of hyperhydric explants was observed on ½ MS + 1mg/l BAP (41.07%) compared with those cultured on 1MS + 1mg/l BAP (18.91%) (Figure 10.D).

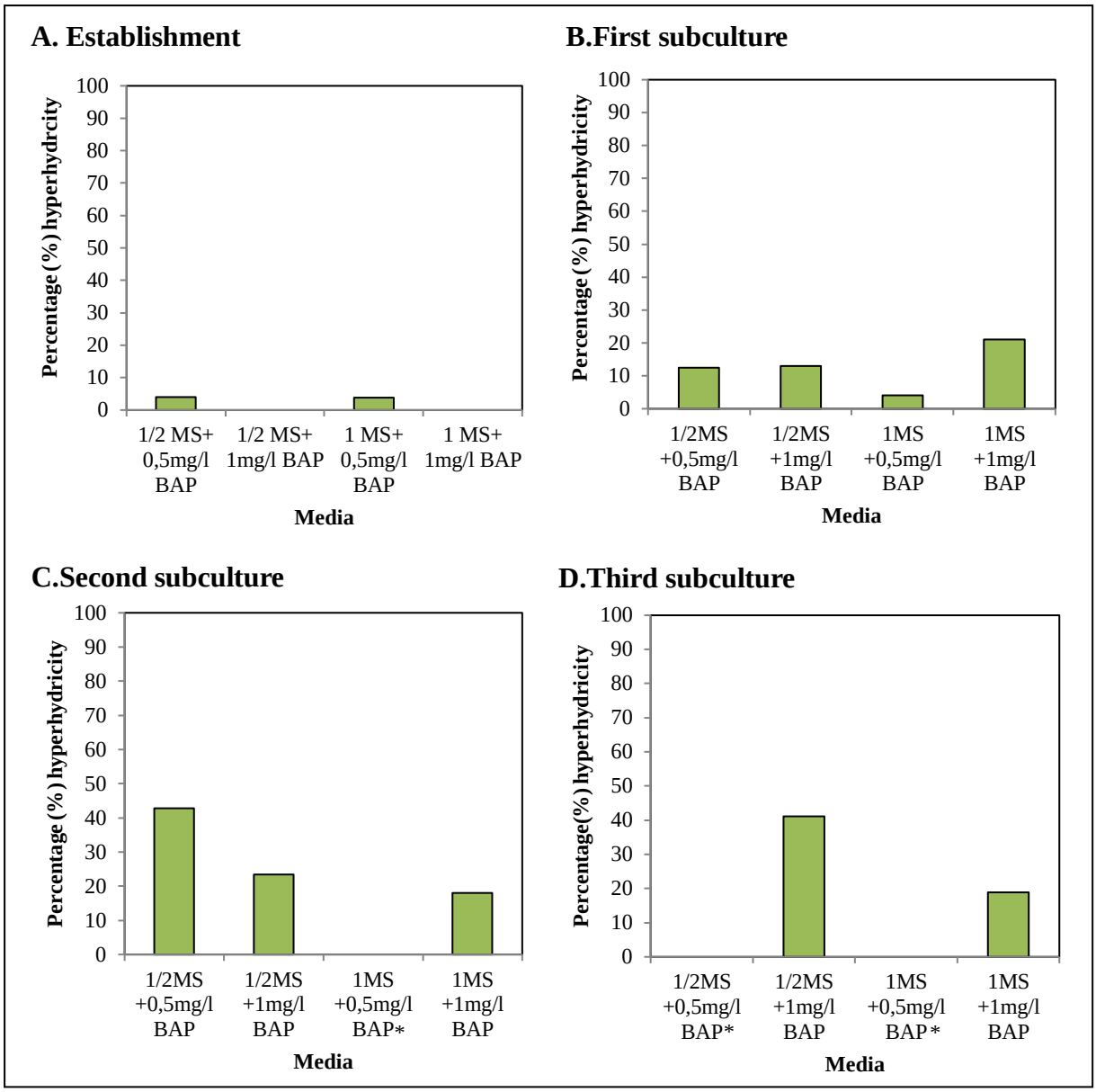


Figure 10. The percentage (%) hyperhydricity observed in *in vitro* avocado axillary bud explants cultured on four tissue culture media within 6 weeks of establishment (A), the first subculture (B), the second subculture (C) and the third subculture (D). There was no relationship between hyperhydricity and media at establishment (*p=0.58*), at the first subculture (*p=0.27*) and at the second subculture (*p=0.04*). There was a relationship between hyperhydricity and media at the third subculture (*p=0.002*). * No samples available.

For explants cultured on ½ MS + 0.5mg/l BAP the percentage of hyperhydric explants increased from 4% after establishment to 42% after the second subculture (Table 9). Similarly, for explants cultured on ½ MS + 1mg/l BAP hyperhydricity increased from establishment (0%) to the third subculture (41.07%) (Table 9). In contrast to this, there was no significant difference in the percentage of hyperhydric explants cultured on the media containing 1MS over the culture period (Table 9).

Table 9. The percentage (%) hyperhydricity observed in *in vitro* avocado axillary bud explants cultured on four tissue culture media within 6 weeks of establishment, the first subculture, the second subculture and the third subculture.

Medium	Percentage (%) hyperhydricity				
	Establishment	First subculture	Second subculture	Third subculture	*p* values
½ MS + 0.5mg/l BAP	4^{a}	12.5^{ab}	42.8^{b}	-	*p=0.0003*
½ MS + 1mg/l BAP	0^{a}	12.96^{ab}	23.37^{ab}	41.07^{b}	*p=0.0001*
1MS + 0.5mg/l BAP	3.84	4	-	-	*p=0.97*
1MS + 1mg/l BAP	0	21.05	18	18.09	*p=0.14*

Lower case alphabets show significant differences amongst subcultures, according to statistical analysis conducted for each medium

2.3.3.4 Number of shoots

From establishment to the third subculture, the number of shoots (Figure 11) produced from explants cultured on the four tissue culture media tested ranged between 0.46 ± 0.08 and 1.08 ± 0.21 (Figure 12, A-D). There was no significant difference in the number of shoots developed across the four media after *in vitro* establishment and the first subculture (Figure 12, A and B). After the second subculture, explants cultured on ½ MS + 1mg/l BAP produced the lowest number of shoots (0.46 ± 0.08) from the media tested (Figure 12.C). After the third subculture, however, there was no significant difference in the number of shoots developed from explants cultured on ½ MS + 1mg/l BAP and 1MS + 1mg/ l BAP (Figure 12.D).

Figure 11. An *in vitro* avocado shoot.

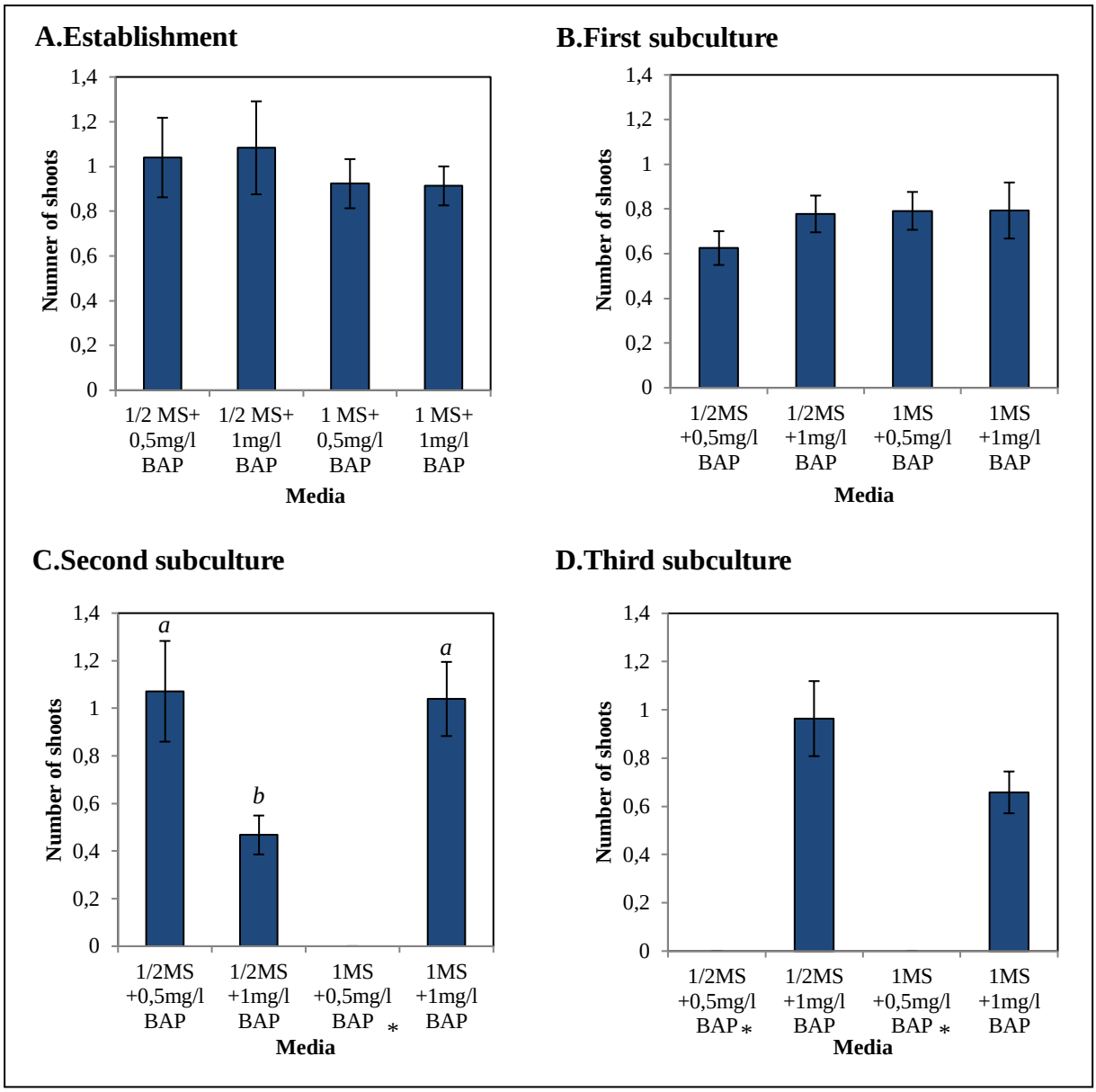


Figure 12. The number of shoots (mean ± SE) that developed from *in vitro* avocado axillary bud explants cultured on four tissue culture media, 6 weeks after establishment (A), the first subculture (B), the second subculture (C) and the third subculture (D). There was no significant difference in number of shoots developed across the media at establishment (*H (3, 97) =0.003, p=1*), at the first subculture (*H (3, 159) = 3.44, p=0.320*) and at the third subculture (*t (91) =1.45, p=0.149)*. There was a significant difference in the number of shoots developed across the media at the second subculture (*H (2, 155) =14.78, p<0.001*). *No samples available

There was no numerical difference in the number of shoots produced between establishment and the third subculture for explants cultured on all the media tested (Table 10).

Table 10. The number of shoots (mean ± SE) that developed from *in vitro* avocado axillary bud explants cultured on four tissue culture media, 6 weeks after establishment, the first subculture, the second subculture and the third subculture.

Medium	Number of shoots				
	Establishment	First subculture	Second subculture	Third subculture	*p* values
½ MS + 0.5mg/l BAP	1.04 ± 0.17	0.64 ± 0.07	1.07 ± 0.02	-	*p=0.06*
½ MS + 1mg/l BAP	1.08 ± 0.20^{a}	0.77±0.08^{a}	0.46±0.08^{b}	0.96±0.15^{a}	*p=0.0003*
1MS + 0.5mg/l BAP	0.92± 0.10	0.79±0.08	-	-	*p=0.35*
1MS + 1mg/l BAP	0.91± 0.08	0.79±0.12	1.03±0.155	0.65±0.08	*p=0.20*

Lower case alphabets show significant differences amongst subcultures, according to statistical analysis conducted for each medium

2.3.3.5 Number of axillary buds

Over the four culture periods the number of axillary buds (Figure 13) produced from explants cultured on the four tissue culture media tested ranged between 2.77 ± 0.27 and 5.25 ± 0.76 (Figure 14, A-D). There was no significant difference in the number of axillary buds developed across the four media after *in vitro* establishment (Figure 14.A) and the first subculture (Figure 14.B). After the third subculture, there was no significant difference in the number of axillary buds developed from explants cultured on ½ MS + 1mg/l BAP compared with those cultured on 1MS + 1mg/l BAP (Figure 14.D).

Figure 13.An *in vitro* avocado axillary bud.

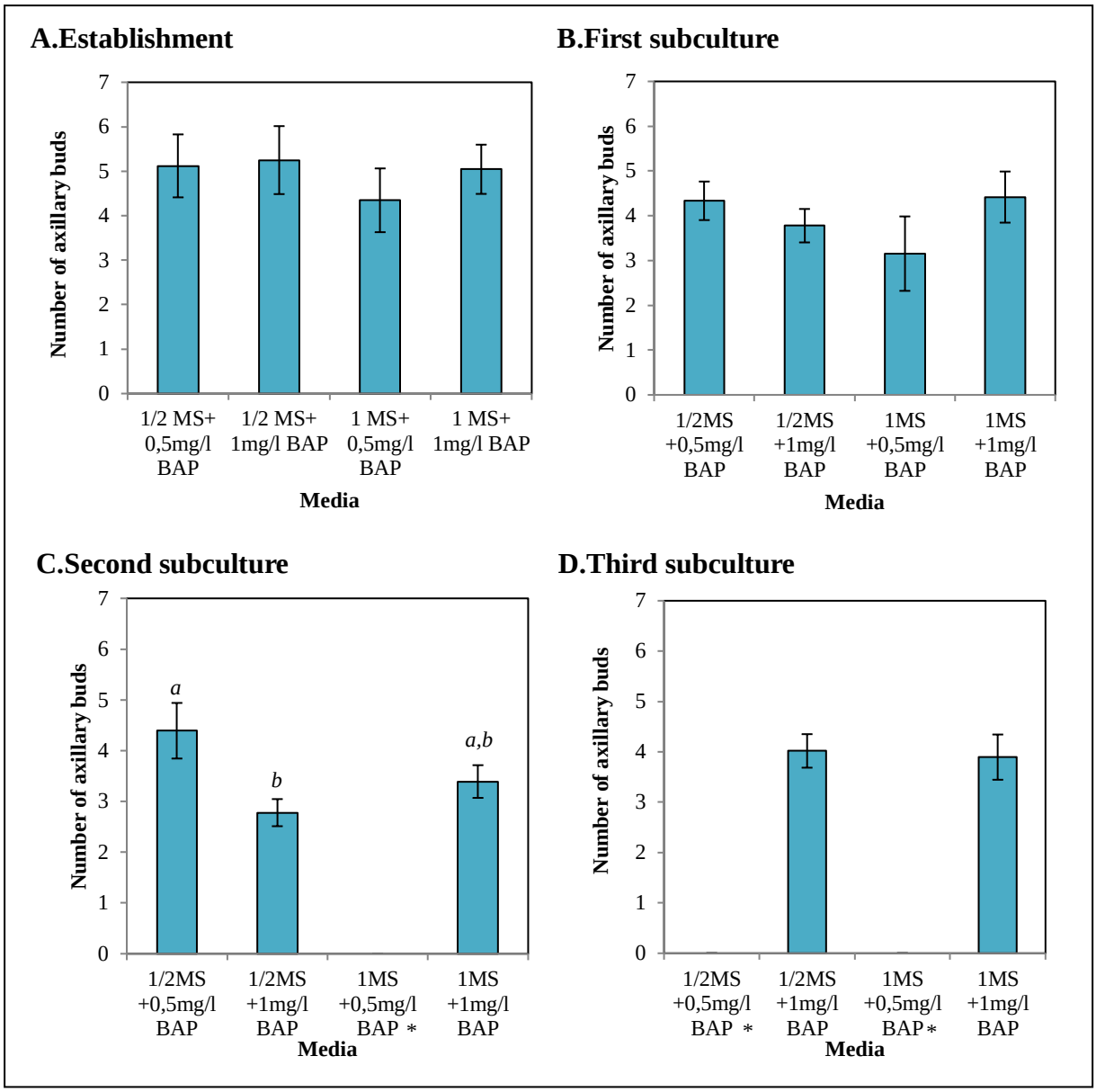


Figure 14. The number of axillary buds (mean ± SE) that developed from *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment (A), the first subculture (B), the second subculture (C) and the third subculture (D). There was no significant difference in the number axillary buds developed between the media at establishment (*H (3, 97) =1.20, p=0.75*), at the first subculture (*H (3, 150) =2.75, p=0.43*) and at the third subculture *(t (91) =0.18, p=0.85).* There was a significant difference in the number of axillary buds developed across the media at the second subculture (*H (2, 155) = 9.16, p=0.01*). *No samples available.

There were fluctuations in the number of axillary buds produced between establishment and the third subculture for explants cultured on all the media tested (Table 11).

Table 11. The number of axillary buds (mean ± SE) that developed from *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment, the first subculture, the second subculture and the third subculture.

Medium	Number of axillary buds				
	Establishment	First subculture	Second subculture	Third subculture	*p* values
½ MS + 0.5mg/l BAP	5.12 ±0.70	4.33±0.42	4.39±0.54	-	*p=0.54*
½ MS + 1mg/l BAP	5.25±0.76^{bc}	3.77±0.37^{abc}	2.77±0.38^{a}	4.01±0.04^{bc}	*p=0.004*
1MS + 0.5mg/l BAP	4.34±0.71	3.15±0.82	-	-	*p=0.31*
1MS + 1mg/l BAP	5.04±0.55	4.41±0.56	3.39±0.32	3.85±0.44	*p=0.12*

Lower case alphabets show significant differences amongst subcultures, according to statistical analysis conducted for each medium

In vitro avocado leaves were observed to be arranged in an opposite pattern, while the leaves on avocado mother plants were arranged in an alternate pattern (Figure 15).

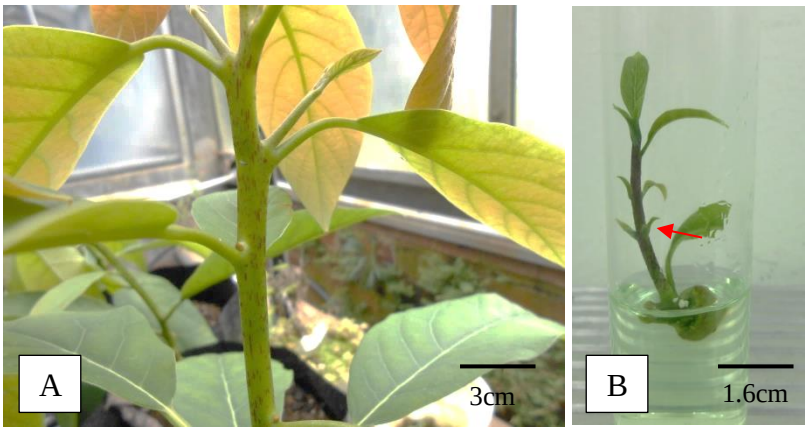


Figure 15. Axillary buds were arranged in an alternate pattern on mother plants (A), while arranged in an opposite pattern on *in vitro* shoots (B).

2.3.3.6 Shoot length

There was no significant difference in shoot length after establishment (Figure 16.A). After the first and the second subculture, explants cultured on 1MS + 1mg/l BAP produced a significantly longer shoot (3.41 ± 0.30 cm) than with those cultured on ½ MS +1 mg/l BAP (1.20 ± 0.20 cm) (Figure 16, B and C).

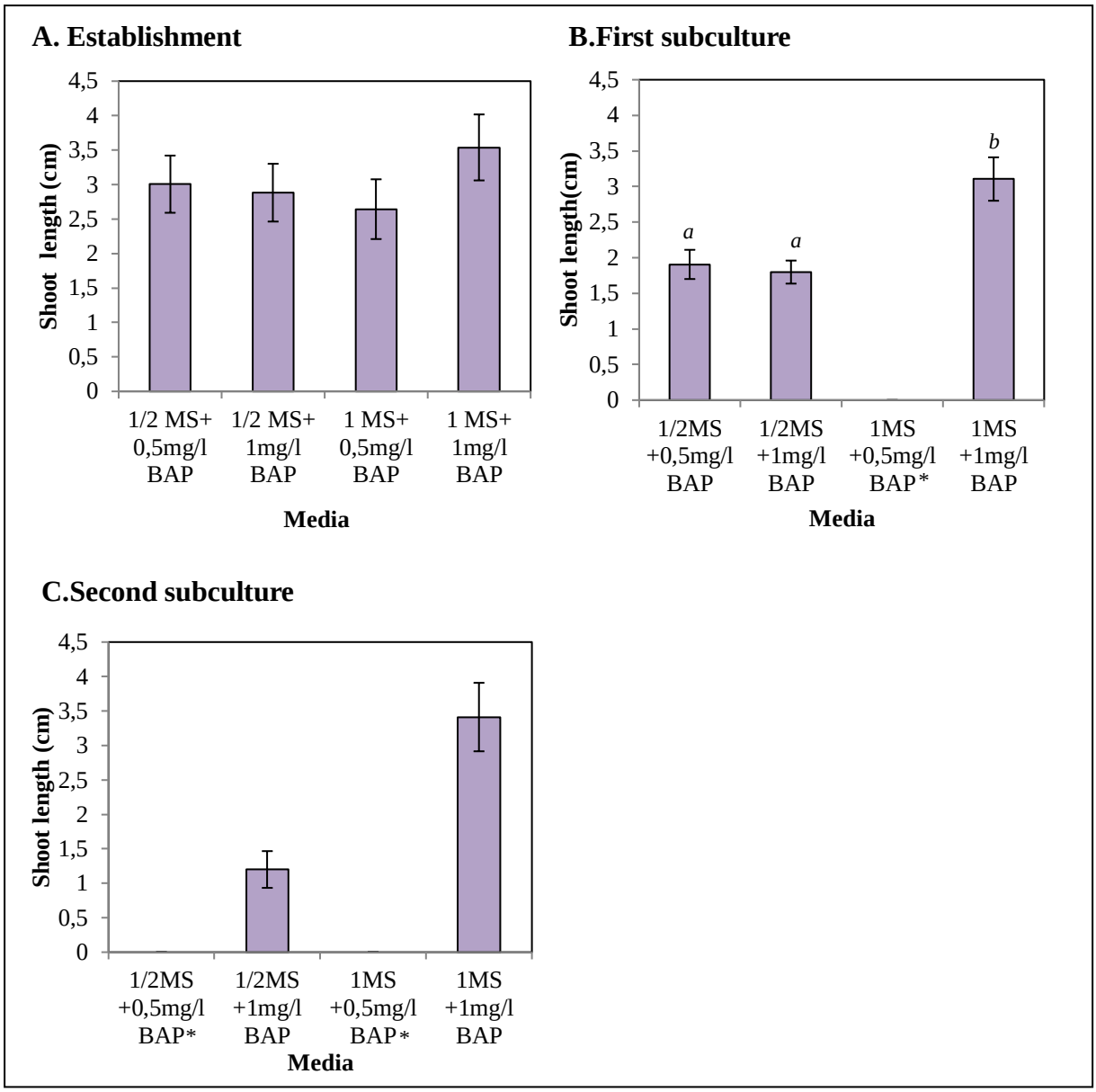


Figure 16. Shoot length (mean ±SE) (cm) of *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment (A), the first subculture (B) and the second subculture (C). There was no significant difference in the length of the shoot developed across the four media tested at establishment *(H (3, 62) =2.52, p=0.47)*. There was however, a significant difference in the length of the shoot developed across the four media at the first subculture (*H (2,82) =13.03, p=0.0015*) and at the second subculture (*t (24) =3.62, p=0.001*). * No samples available.

Taller shoots were produced at establishment (1.04 ± 0.17 cm) than at the first subculture (0.62 ± 0.07 cm) for explants cultured on ½ MS + 0.5mg/l BAP (Table 12). Similarly, for explants cultured on ½ MS + 1mg/l BAP taller shoots developed at establishment (2.88 ± 0.41 cm) than at the second subculture (1.2 ± 0.26 cm) (Table 12). In contrast to this, shoot

length remained the same for explants cultured on 1MS + 1mg/l BAP from establishment to the second subculture (Table 12).

Table 12. Shoot length (mean ±SE) (cm) of *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment, the first subculture and the second subculture.

Medium	Shoot length(cm)			
	Establishment	First subculture	Second subculture	*p* values
½ MS + 0.5mg/l BAP	1.04±0.17 ^{a}	0.62±0.07 ^{b}	-	*p=0.01*
½ MS + 1mg/l BAP	2.88±0.41^{a}	1.79±0.16^{ab}	1.2±0.26^{b}	*p =0.0015*
1MS + 0.5mg/l BAP	2.64±0.43	-	-	-
1MS + 1mg/l BAP	3.53±0.47	3.10±0.30	3.41±0.49	*p =0.86*

Lower case alphabets show significant differences amongst subcultures, according to statistical analysis conducted for each medium

2.3.3.7 Callus formation

Callus with different textures and colours was observed (Figure 17). While this was an overall observation, no specific data was recorded to determine whether a certain type of callus (e.g. friable callus or dense white callus) was commonly associated with any of the media tested. Callus was observed to develop mainly at the proximal cut surface of explants (Figure 17).

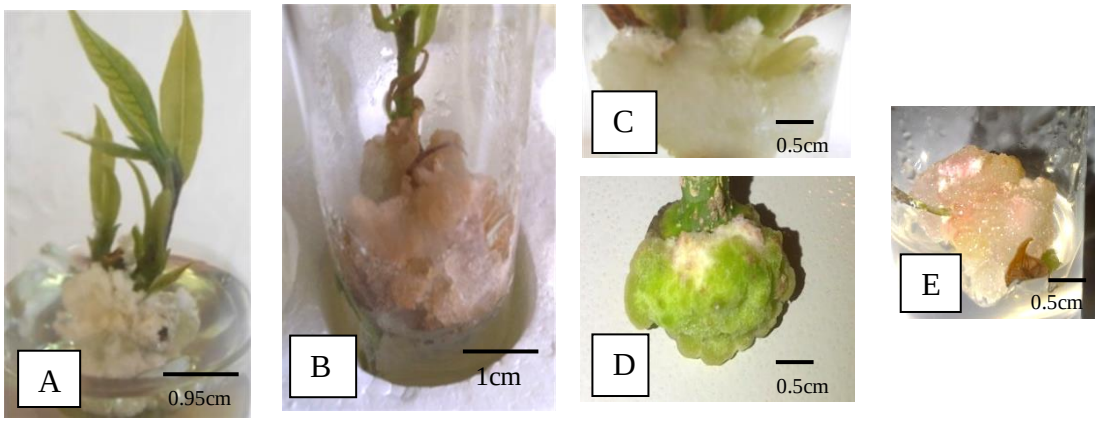


Figure 17. Observations made regarding different types of callus formation in *in vitro* avocado explants. A. friable white callus, B. orange-brown callus, C. dense white callus, D. green callus and E. pink callus.

Callus formation scores were less than score 2 over all the culture periods (Table 4, Figure 18, A-D). There was no significant difference between the callus formation scores across the

media after establishment (Figure 18.A). After the first, the second and the third subculture callus formation was observed to be significantly higher on explants cultured on 1MS + 1mg/l BAP compared with explants cultured on ½ MS + 1mg/l BAP (Figure 18, B-D).

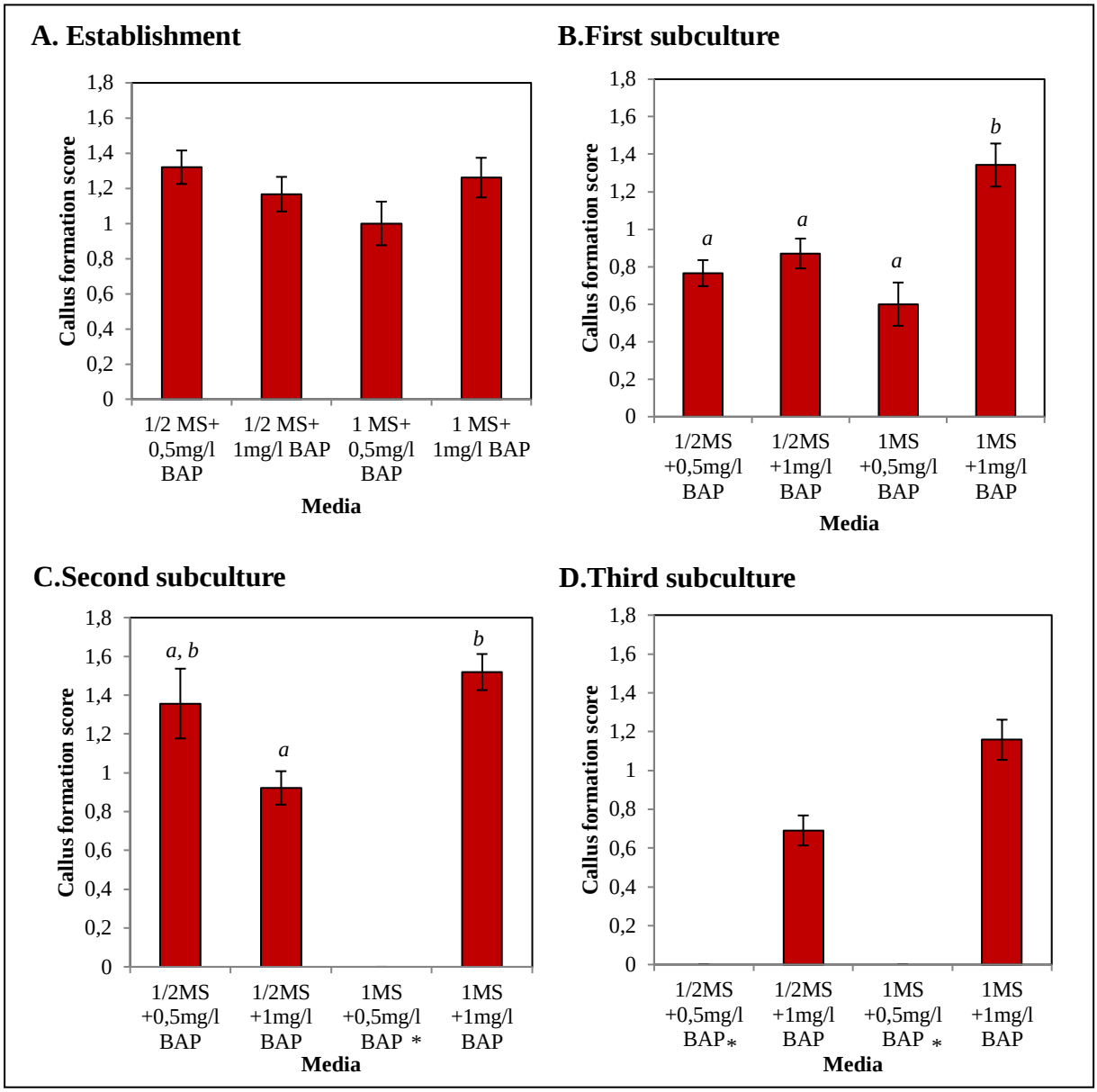


Figure 18. Callus formation score (mean ± SE) of *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment (A), the first subculture (B), the second subculture (C) and the third subculture (D). There was no significant difference in the callus formation scores across the four media at establishment (*H (3, 98) = 4.41, p=0.22*). However, there was a significant difference in the callus formation scores across the four media tested at the first subculture (*H (3, 181) = 24.01, p<0.0001*), at the second subculture (*H (2,156) =18.7, p<0.0001*) and at the third subculture (*t (91) = 3.95, p=0.002*). * No samples available.

There was an overall decrease in callus formation from establishment to the first subculture for all the media tested except for 1MS + 1mg/l BAP (Table 13).

Table 13. Callus formation score (mean ± SE) of *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment, the first subculture, the second subculture and the third subculture.

Medium	Callus formation score				
	Establishment	First subculture	Second subculture	Third subculture	*p* values
½ MS + 0.5mg/l BAP	1.32±0.09^{a}	0.76±0.06^{b}	1.35±0.17^{a}	-	*p=0.0001*
½ MS + 1mg/l BAP	1.16±0.09^{a}	0.87±0.07^{ab}	0.92±0.08^{ab}	0.69±0.07^{b}	*p=0.01*
1MS + 0.5mg/l BAP	1.00±0.12^{a}	0.60±0.11^{b}	-	-	*p=0.02*
1MS + 1mg/l BAP	1.26±0.11	1.34±0.11	1.51±0.09	1.15±0.10	*p =0.05*

Lower case alphabets show significant differences amongst subcultures, according to statistical analysis conducted for each medium

2.4 DISCUSSION

2.4.1 Stage 0. Mother plant establishment and pre-treatment

The observations suggested that the soil which was used to cultivate the mother plants was not suitable, as it waterlogged easily even with the inclusion of perlite and the control of irrigation. Avocado plants require aerated soils for healthy development and consequently cannot grow in waterlogged soils (Dann *et al.*, 2013; Menge and Ploetz, 2003; Ho and Zentmyer, 1977). Additionally, root rot disease (see 3.1.2) develops in these soils (Dann *et al.*, 2013; Menge and Ploetz, 2003; Ho and Zentmyer,1977). Root rot is a major pathogen affecting many avocado varieties, of which cv. 'Edranol' is highly susceptible (Snyman *et al.*, 1984). The physiological observations indicated that the avocado mother plant had developed root rot (Dann *et al.*, 2013; Menge and Ploetz, 2003; Ho and Zentmyer, 1977). This suggested that the regular application of the systematic fungicide Ridomil Gold alone was not effective in controlling the disease. The high percentage of avocado mother plants which had died during establishment demonstrated the need for further experiments aimed at optimising mother plant establishment, in order to maintain healthy plant material for subsequent experimentation. This is discussed later in Chapter Three.

2.4.2 Stage 1. Establishment of an aseptic culture

2.4.2.1 Contamination

In this study the contamination of explants was observed from establishment through to the subsequent subcultures (Figure 6). It is generally expected that contamination decreases with time in culture, as at establishment the explants originate from the external environment, and are thereafter exposed to a sterile environment (Kane, 2016; Moreno-Vázquez *et al.*, 2014; Greenwood, 1995; Reed and Tanprasert, 1995; Leifert *et al.*, 1991). However, it may be possible that the contamination of explants would increase *in vitro* as this stress-inducing environment may make the explants more susceptible to pathogens which do not usually affect them in nature (Habiba *et al.*, 2002; Bradbury, 1970). Factors which may make the *in vitro* environment stress-inducing for explants include the high humidity in the culture container and the components of the tissue culture medium (Van Staden *et al*. 2004). The response of the avocado explants (Figure 6) suggested that endogenous contaminants were still present in the tissues (Kane, 2016; Ryan, 1994; Leifert *et al.*, 1991). As a result, cv. 'Edranol' mother plants would need to be further pretreated to eliminate the systemic pathogens which may have caused the *in vitro* contamination of explants (George, 2008; Cassells, 1997).

The explants cultured on the different media did not respond in the same way in terms of contamination over the subsequent subcultures (Figure 6). Contamination appeared to be associated with the media containing 0.5mg/l BAP (Figure 6, B and C). Explants that are weaker may be more susceptible to contamination than explants that are stronger (Greenwood, 1995). Considering this, it is possible that an imbalance in plant growth regulators in the explants cultured on 0.5mg/l BAP may have led to them being weak, and thus more susceptible to contamination than those cultured on 1mg/l BAP (Kane, 2016; George and Deberg, 2008; Greenwood, 1995; Leifert *et al.*, 1991).

2.4.3 Stage 2. *In vitro* establishment and multiplication

To develop an *in vitro* system for the proliferation of axillary buds for avocado cv. 'Edranol', four tissue culture media were tested, comprising of MS and BAP in two concentrations. In order for multiplication of axillary buds to occur *in vitro*, more explants need to be produced than lost at each subculture. The observation that there were no viable explants from those cultured on the media containing 0.5mg/l BAP by third subculture (Table 5) suggested that this concentration of BAP was not conducive for growth and development for cv. 'Edranol',

and thus promoted contamination *in vitro* (Figure 6), death (Figure 8) and hyperhydricity (Figure 10).

2.4.3.1 Tissue browning and death

In this study, apical necrosis was not observed as has been observed in some other avocado varieties (Bairu *et al.*, 2009; Kataeva *et al.*, 1991), but instead, browning was observed throughout explants (Table 5). This suggested that the explants of cv. 'Edranol' cultured on the media tested in this study did not experience the conditions which promote apical necrosis, such as iron and boron (both of which are supplied in MS) deficiencies (Bairu *et al.*, 2009; Barghchi and Alderson, 1996; Kataeva *et al.*, 1991). Tissue browning, as was observed in this study (Figure 7), is not desirable for *in vitro* productivity. In light of this, it is possible that the use of antioxidants would be beneficial for reducing tissue browning of cv.'Edranol' explants (Ahmed *et al.*, 2001; Dalsaso and Guevara, 1989).

After *in vitro* establishment, tissue browning was observed only in the explants cultured on the media containing 0.5mg/l BAP (Figure 7.A) This suggested that surface decontamination with 1% sodium hypochlorite did not promote browning, but instead the concentration of plant growth regulators was involved in the tissue browning of explants (Bairu *et al.*, 2009; Kataeva *et al.*, 1991). Further observations suggested that tissue browning and death was related to the media containing 0.5mg/l BAP (Figure 7). There was no difference in the tissue browning and death of explants cultured on the media containing 1mg/l BAP after all subcultures (Figure 7 and Figure 8) which further suggested that the concentration of BAP was more important in triggering browning and subsequent death than the concentration of MS (Bairu *et al.*, 2009; Kataeva *et al.*, 1991). For both media containing 0.5mg/l BAP, death was observed to be significantly higher at the first and second subcultures, respectively, compared with at establishment (Table 8). This suggested that these media promoted the death of explants with each subculture. This may have come about through a decline in the physiological state of the explants through subsequent subcultures, causing explants to become weaker (George and Deberg, 2008; Trippi, 2012). Additionally, particularly in woody species the excision of explants at subculture has been observed to result in the release of phenolic compounds (Minocha and Jain, 2000; Ahmad *et al.*, 2013). Thus, insufficient cytokinin combined with the secondary metabolism induced as a result of wounding at subculture may have contributed to an increase in tissue browning and subsequent death of the explants cultured on 0.5mg/l BAP (Kane, 2016; Ahmad *et al.*, 2013).

The results of this study were similar to Nhut *et al.* (2008) who observed browning and necrosis associated with explants cultured on 0.5mg/l BAP. However, in contrast to what was observed in this study, in the GA-13 avocado rootstock necrosis was observed with 1mg/l BAP (Pliego-Alfaro, 1988). These observations suggested that each variety of avocado responds differently to the BAP concentrations in the tissue culture medium in terms of tissue browning and death (Barceló-Muñoz *et al.*, 1999; Castro *et al.*, 1995).

With regard to the relationship between PGRs and tissue browning, in other varieties of avocado tissue browning was observed in explants cultured on media which were free of cytokinin (Bandaralage *et al.*, 2015; Ahmed *et al.*, 1997). Thus, it may be possible that 0.5mg/l BAP was too low a concentration for the growth and development of cv. 'Edranol' explants, and as a result explants did not respond favourably to this treatment. Studies have shown that there are relationships between PGR levels and the uptake and use of nitrogen (Krouk *et al.*, 2011). It was possible that the concentration of BAP affected the way that the explants interacted with nitrogen in the medium, which would have affected how the explants subsequently responded (Kiba *et al.*, 2011). However, the relationship between BAP and the uptake of nitrogen from the medium in cv. 'Edranol' would have to be experimentally tested.

The presence of tissue browning (Figure 7) of *in vitro* tissues may be due to a variety of reasons; tissue browning may come about as a wounding response, as a result of the oxidation of phenolic compounds (Ahmad *et al.*, 2013). The results of this study suggested that browning and subsequently death *in vitro* was caused by insufficient balance of plant growth regulators (Bairu *et al.*, 2009; Kataeva *et al.*, 1991), but this would need to be verified. In other varieties of avocado, however, the concentration of MS was observed to be important in the browning of explants, with increased oxidation being observed at higher concentrations of MS (Biasi *et al.*, 1994). This is because the browning occurs as a result of secondary metabolism, which is related to nitrogen and carbon metabolism (Wojtania *et al.*, 2015). It has been observed that the interactions between BAP, nitrogen and carbon may bring about different phenolic responses across species, and in some cases, such as magnolia, it differs within a species (Wojtania *et al.*, 2015). Thus, the response of explants to the tissue culture medium in terms of tissue browning and death may vary amongst the different varieties of avocado (Fuentes *et al.*, 2004; Castro *et al.*, 1995).

2.4.3.2 Hyperhydricity

Hyperhydric avocado explants were deformed and did not exhibit normal growth (Figure 9). There appeared to be no relationship between media and hyperhydricity from establishment to the third subculture (Figure 10). However, for the explants cultured on the media containing ½ MS, hyperhydricity was observed to be significantly higher at the third/second subculture than at establishment, while there was no significant difference in hyperhydricity observed with the explants cultured on 1MS over the culture period (Table 9). This suggested that ½ MS promoted increased hyperhydricity with time in culture. These findings were similar to that of Reed *et al.* (2013) who determined that higher incidences of hyperhydricity occurred at lower mineral concentrations in pear (*Pyrus communis*) explants. Thus, it may be possible that the explants of cv. 'Edranol' cultured on ½ MS displayed increased hyperhydricity as a result of the low nutrient concentrations in the medium compared with 1MS (Hazarika, 2006; de la Viña *et al.*, 2001; Ziv, 1991; Daguin and Letouze, 1986). The interactions of minerals, nitrogen and BAP in avocado explants, however, would have to be experimentally verified.

In contrast to what was observed in this study, other studies determined that hyperhydricity occurred more frequently at high cytokinin and high nitrogen concentrations, and that the use of cytokinins, particularly BAP, increased hyperhydricity with time in culture (Badr-Elden *et al.*, 2012; Barceló-Muñoz and Pliego-Alfaro, 2012; de la Viña *et al.*, 2001; Kataeva *et al.*, 1991; Schall, 1987; Daguin and Letouze, 1986). Higher levels of nitrogen promoted the synthesis and accumulation of BAP in tissues, thus higher levels of hyperhydricity were seen (Martins *et al.*, 2015; Wu *et al.*, 2011; Debergh *et al.*, 1992; Debergh, 1983). In light of this, Pliego-Alfaro *et al.* (1987) attributed hyperhydricity in avocado to high levels of endogenous BAP. However, the response of cv. 'Edranol' explants in terms of axillary bud and shoot development suggested low levels of endogenous BAP (see 2.4.3.3 below).

2.4.3.3 The number of shoots and axillary buds

Plant material needs to be supplemented with the correct concentration of nutrients and plant growth regulators in order to achieve optimum growth and development *in vitro* (Barceló-Muñoz and Pliego-Alfaro, 2012; George and Deberg, 2008; Razdan, 2003; Dodds and Roberts, 1985). There was no difference in the number of shoots developed on all the media (Figure 12) which indicated that the explants were not responding to the culture media but instead to other factors, such as endogenous hormones (da Costa *et al.*, 2013; Barceló-Muñoz

and Pliego-Alfaro, 2012; Desjardins *et al.*, 2007; Castro *et al.*, 1995). Irrespective of media, avocado explants showed strong apical dominance as not more than one shoot was produced (Figure 12). Similarly, in other studies it was observed that avocado explants cultured with different concentrations of BAP all showed apical dominance (Castro *et al.*, 1995; Harty, 1985). Apical dominance is not favourable, however, as it limits *in vitro* productivity.

In other varieties of avocado between 1 and 4.8 shoots per explant developed on the medium selected as the most appropriate (Zulfiqar *et al.*, 2009; Nhut *et al.*, 2008; Schaffer *et al.*, 1999; Ahmed *et al.*, 1997; Barringer *et al.*, 1996; Mora-Aviles, 1994; Zirari and Lionakis, 1994; Castro *et al.*, 1995; Vega, 1989; Cooper, 1987). The responses of cv.'Edranol' explants in terms of shoot production (Figure 12) were within the range observed for other varieties. However, in cv.s 'Fuerte', 'Colin V-33', 'Hass' and 'Duke' more than one shoot per explant was produced, suggesting that the tissue culture media for cv. 'Edranol' requires further improvement (Zulfiqar *et al.*, 2009; Nhut *et al.*, 2008; Schaffer *et al.*, 1999; Ahmed *et al.*, 1997; Barringer *et al.*, 1996; Mora-Aviles, 1994; Zirari and Lionakis, 1994; Castro *et al.*, 1995; Vega, 1989; Cooper, 1987).

The response of the explants in terms of shoot growth suggested that endogenous auxin concentrations may have been higher than the concentrations of cytokinin (BAP) tested in this study, thus apical dominance and callus formation (see Section 2.4.3.5) was observed (Barceló-Muñoz and Pliego-Alfaro, 2012; Zulfiqar *et al.*, 2009; Moshkov *et al.*, 2008). High endogenous auxin production has been linked with ethylene release, the accumulation of which has been associated with slow growth and development of avocado plants *in vitro* (Moshkov *et al.*, 2008; Matthys *et al.*, 1995; Schall, 1987). Ethylene release also occurs as a wounding response of *in vitro* tissues (Moshkov *et al.*, 2008; Matthys *et al.*, 1995; Schall, 1987). Thus, it may be possible that ethylene production reduced the growth and development of the *in vitro* avocado explants and contributed to tissue browning (Barceló-Muñoz and Pliego-Alfaro, 2012; Zulfiqar *et al.*, 2009; Barrera-Guerra *et al.*, 2001; Schall, 1987). However, the endogenous auxin concentrations and ethylene production in *in vitro* avocado explants will need to be experimentally verified.

The number of axillary buds which developed did not differ significantly between explants cultured on the four media tested after establishment, the first and the third subculture (Figure 14). Amongst the subcultures there was no significant difference in the number of axillary buds developed for each medium (Table 12). In other varieties of avocado, between 3 to 11

axillary buds per culture were obtained on the selected medium (de la Viña *et al.*, 2001; Barceló-Muñoz *et al.*, 1999; Pliego-Alfaro, 1988). In this study, between 2.77 ± 0.27 and 5.25 ± 0.76 axillary buds per explant developed on all the media tested (Figure 13). This meant that the number of axillary buds was within the range observed for other avocado varieties. However, for the purpose of mass multiplication of axillary buds, this amount would not be sufficient. Thus, the media tested in this study were sufficient for initiation of cultures, but not for the continued multiplication of shoots and axillary buds (Zulfiqar *et al.*, 2009; Nhut *et al.*, 2008; Schaffer *et al.*, 1999; Ahmed *et al.*, 1997; Barringer *et al.*, 1996; Zirari and Lionakis, 1994; Castro *et al.*, 1995; Vega, 1989; Cooper, 1987).

The response of cv. 'Edranol' explants in terms of axillary bud and shoot development suggested that a higher concentration of BAP than 1mg/l is required to break apical dominance and increase axillary bud proliferation (Zulfiqar *et al.*, 2009; Nhut *et al.*, 2008; Schaffer *et al.*, 1999; Ahmed *et al.*, 1997; Barringer *et al.*, 1996; Zirari and Lionakis, 1994; Castro *et al.*, 1995; Vega, 1989; Cooper, 1987). For some varieties of avocado, for instance, cv.s 'Bacon' and 'Mantequilla' (López *et al.*, 2015; Dalsaso and Guevara, 1989), 2mg/ l BAP was chosen as the optimum concentration of cytokinin. 2mg/l BAP was also selected as the optimum concentration for the *in vitro* propagation of other woody species such as *Persea indica* (Nel and Kotze, 1982) and Macadamia (*Macadamia integrifolia*) (Gitonga *et al.*, 2008). However, the use of concentrations of BAP higher than 3mg/l over subsequent subcultures has been observed to cause reduced shoot proliferation and shoot size in other avocado varieties, and has been associated with hyperhydricity in explants (Barceló-Muñoz and Pliego-Alfaro, 2012; Van Staden *et al.*, 2008; Nel and Kotze, 1982). Thus, the optimum concentration of BAP for cv.'Edranol', which would be between 1 to 3mg/l, will need to be experimentally determined in future studies.

2.4.3.4 Shoot length

Explants cultured on 1MS + 1mg/l BAP were observed to produce the longest shoots compared with explants cultured on the other media tested (Figure 16). Shoot elongation is mediated by auxins and gibberellins (Machakova *et al.*, 2008; Razdan, 2003). In other avocado tissue culture studies shoot length of 2-4 cm was observed and the length of shoots observed in this study was within this range (Figure 16) (Zulfiqar *et al.*, 2009; de la Viña *et al.*, 2001; Barceló-Muñoz *et al.*, 1999; Vega, 1989; Pliego-Alfaro and Murashige, 1987).

The nitrogen concentration in the medium (MS) affects growth and morphology of plant tissues (Kane, 2016; Reed *et al.*, 2013; Machakova *et al.*, 2008). The response of the explants in terms of shoot length indicated that ½ MS may not have been suitable for cv. 'Edranol', as from establishment to the subsequent subcultures shoot length was observed to decrease for explants cultured on the media containing ½ MS, while it remained the same for explants cultured on 1MS + 1mg/l BAP (Table 12).

In vitro axillary buds were arranged in an opposite pattern (Figure 15.B), while avocado mother plants exhibit leaves arranged in an alternate pattern (Wolstenholme *et al.*, 2013) (Figure 15.A). This arrangement suggested that *in vitro,* the axillary buds were arranged in an alternate pattern, but the buds on parts of the stem that had not elongated had an opposite appearance (Barceló-Muñoz and Pliego-Alfaro, 2012; Zulfiqar *et al.*, 2009; Pliego-Alfaro and Murashige, 1987). As has been suggested previously (Section 2.4.3.3), ethylene release may have reduced growth and the elongation of stem pieces (Barrera-Guerra *et al.*, 2001). Thus it may be possible that cv. 'Edranol' explants have the potential produce longer shoots. When considering shoot length, it may be more desirable to have a long shoot, as at subculture each axillary bud would be associated with a longer stem section, allowing for easier dissection (Razdan, 2003). Considering this, the addition of GA_3 might result in shoot elongation through internodal extension and play a role in creating the correct balance of plant growth regulators required for optimum growth and proliferation of axillary buds (Kane, 2016; Little and MacDonald, 2003; Shimizu-Sato and Mori, 2001). In other varieties of avocado GA_3 has been used in concentrations ranging from 0.1mg/l to 2mg/l (Bandaralage *et al.*, 2015; Sánchez-Romero *et al.*, 2007; Raharjo and Litz, 2005; del Sol *et al.*, 2000; Vega, 1989; Nel *et al.*, 1983; Young, 1983; Nel and Kotze, 1982). Gibberellic acid, however, was observed to induce explant oxidation in cv. 'Fuerte' (Dalsaso and Guevara, 1989), thus for cv. 'Edranol' the optimum concentration of gibberellic acid for axillary bud proliferation will need to be experimentally determined.

2.4.3.5 Callus formation

Different types of callus (Figure 17) have also been observed in other avocado varieties *in vitro* (Young, 1983), for instance, green and brown callus was observed in cv. 'Gvaram 13' (Schaffer *et al.*, 1999), while green callus was observed in cv.s 'Lula' and 'Waldin' (Young, 1983). In this study, callus was found at the base of stems and was often observed to almost fill the bottom of the test tube (Figure 17). Callus formation at the proximal cut end of

explants has also been observed in other avocado varieties (Young, 1983; Schroeder, 1976, 1973). This production of callus further suggests that cv. 'Edranol' may produce higher levels of endogenous auxin, as callus formation occurs at a high auxin to cytokinin ratio (Barceló-Muñoz and Pliego-Alfaro, 2012; Zulfiqar *et al.*, 2009; Moshkov *et al.*, 2008).

Callus formation was higher in explants cultured on 1MS + 1mg/l BAP at the first, the second and the third subculture (Figure 18). It was observed in other studies that nutrient levels, vitamins and minerals in the culture medium affected callus formation (Reed *et al.*, 2013; Chauhan and Kothari, 2004; Kintzios *et al.*, 2004, 2001). Thus, it was possible that the combination of 1MS and 1mg/l BAP promoted the highest callus formation in cv. 'Edranol' explants. Similar to what was observed in this study, callus formation was observed using 1mg/l BAP in cv.s 'Lula' and 'Waldin' (Young, 1983), and an increase in callus formation was observed as the concentration of BAP increased in avocado embryonic axes (Barrera-Guerra *et al.*, 2001; Barringer *et al.*, 1996).

On average over all the subcultures, callus formation did not exceed score 2 (Table 4) on 1MS + 1mg/l BAP (Figure 16). Thus, callus formation did not limit shoot and axillary bud productivity as axillary buds which are covered in callus cannot be used for experimentation. However, callus formation is not desirable as it may lead to the formation of adventitious shoots which may behave unpredictably in culture (Ikeuchi *et al.*, 2013; George and Deberg, 2008; Razdan, 2003; Dodds and Roberts, 1985).

2.4.4 Conclusion and recommendations

The findings of this study indicated that the tissue culture system for avocado cv. 'Edranol' required further improvement. This should include optimisation of the entire protocol, starting with the establishment and pretreatment of the mother plants, and further improving the tissue culture medium. A combination of strategies could be beneficial in the development of a system for the *in vitro* propagation of avocado cv. 'Edranol'. These improvements include that:

- mother plant establishment should be optimised by considering strategies to eradicate internal contaminants, with a focus on root rot disease
- antioxidants should be used to control tissue browning
- concentrations of BAP from 1 to 3mg/l should be tested
- concentrations of GA_3 from 0.1 to 2mg/l should be tested

3 CHAPTER THREE - Avocado Mother Plant Establishment and Preparation

3.1 INTRODUCTION

3.1.1 Stage 0 preparations

To develop a system for propagating avocados through tissue culture the first step has to be the optimisation of stage 0, which is mother plant establishment and pre-treatment. By doing this, a more favourable response of the material to the *in vitro* environment may be achieved. For *in vitro* vegetative propagation, researchers have used starting material from avocado mother plants that were growing in the field (in orchards) and in containers (Bandaralage *et al.*, 2015; Sanclemente *et al.*, 2013; Zulfiqar *et al.*, 2009; Neuhaus *et al.*, 2007; Barrera-Guerra *et al.*, 2001; Pliego-Alfaro *et al.*, 1987). The benefit of using container-grown material instead of field-grown material is that the plants can be more rigorously pretreated to promote vigorous growth and to reduce contaminants. Additionally, the growing conditions of plants in containers can be controlled.

3.1.2 Avocado root rot

Phytophthora root rot, caused by *Phytophthora cinnamomi*, is amongst the most virulent of pathogens affecting avocado plants worldwide. *P. cinnamomi* is a water-mould that develops zoospores in waterlogged soils which then thrive in moist and aerated soils (Dann *et al.*, 2013). The fungus affects and destroys the feeder roots of plants, causing them to be blackened and undeveloped (Dann *et al.*, 2013; Menge and Ploetz, 2003; Ho and Zentmyer, 1977). Other physiological signs of root rot include leaf drop and stem-die back (Dann *et al.*, 2013; Menge and Ploetz, 2003; Ho and Zentmyer, 1977). There is currently no cure for root rot, thus a combination of strategies have been used to control and prevent the infection by *P. cinnamomi* of avocado roots, including careful site selection, the use of resistant rootstocks, the control of irrigation and the use of nutrients, chemicals and fungicides (Dann *et al.*, 2013; Menge and Ploetz, 2003; Coffey, 1987; Ho and Zentmyer, 1977).

3.1.3 Containers and soil for avocado propagation

There are three main components that are used to classify soil types, namely clay, sand and silt (Evett *et al.*, 2008; USDA, n.d.). The soil triangle is used to classify soil types based on the percentage of clay, sand and silt that is contained in a soil sample (Figure 19). Each plant

species has different preferences for soil type, as different soil types vary in their physical and chemical properties.

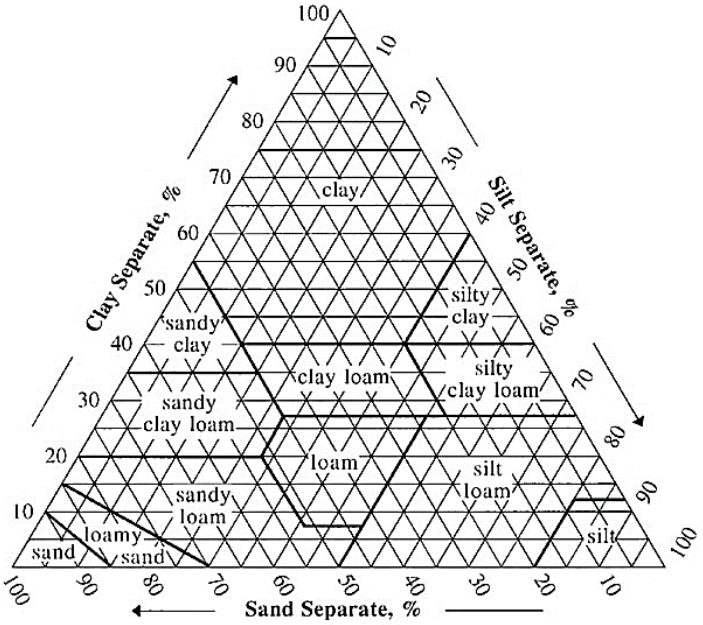


Figure 19. The soil triangle which is used to classify soil type based on the percentage of sand, clay and silt that is contained in a soil sample (USDA, n.d.).

In avocado orchards, sand is the biggest component of the soil (Wolstenholme, 2013; Claassens and Welgemoed, 2011). For propagation purposes, sandy soils are not suitable, as they are heavy to transport and they dry out very rapidly because they do not hold moisture (Landis and Morgan, 2009; Bilderback, 1982). As a result, different soils and materials have been used to propagate avocados (Claassens and Welgemoed, 2011). The general characteristics of a good avocado propagating medium is one that is aerated, moist and free-draining (Claassens and Welgemoed, 2011).

3.1.4 Soil characteristics

3.1.4.1 Physical characteristics

A combination of physical and chemical soil characteristics determines how well a plant grows. Physical soil characteristics include bulk density, water holding capacity, porosity and air-filled porosity. The bulk density of soil refers to the mass of the soil per unit volume (Crowley, 2007). Bulk density can be used as a measure of the amount of pore spaces that soils have, how much water soils can hold and the extent of soil aeration (Crowley, 2007; Ferreyra *et al.*, 2007; Kozlowski, 1999). For instance, with two soils that have the same

volume but different mass, the soil with the higher mass will have a higher bulk density, a lower water holding capacity and will be less aerated than the soil with a low bulk density (Crowley, 2007; Ferreyra *et al.*, 2007; Kozlowski, 1999). Bulk density also influences how easily roots are able to grow in the soil, as a plant root will have to exert more force to penetrate through soils with high bulk densities (Crowley, 2007; Ferreyra *et al.*, 2007; Kozlowski, 1999).

Soil water holding capacity (also known as field capacity or pot/container capacity) refers to the amount of water that a soil can hold, after gravitational water has drained away (House, 2011; Bilderback, 1982; Gessert, 1976). Soils at field capacity contain both water and air spaces and porosity refers to the total pore spaces in a soil, i.e. pore spaces that are filled with both air and water (House, 2011; Bilderback, 1982; Gessert, 1976). Wilting point is reached when plants cannot access water from the soil. The difference between field capacity and wilting point signifies the amount of water in the soil that is available to plants (plant available water) (House, 2011; Zotarelli *et al.*, 2010), and this amount of water is dependent on soil texture (Figure 20). The wilting point of sandy loam soil is at about 0.06m^3/m^3 or 6% soil water content and field capacity is at about 20% soil water content (Figure 20). In terms of its physical properties, the soil that is best for growing avocados has a low bulk density (0.5 – 0.8 g/cm^3), high aeration (15 to 25%) and porosity of about 46% (Claassens and Welgemoed, 2011; Salgado and Cautın, 2008; Crowley, 2007; Ferreyra *et al.*, 2007; Kozlowski, 1999; Salazar-Garcia and Cortes-Flores, 1986).

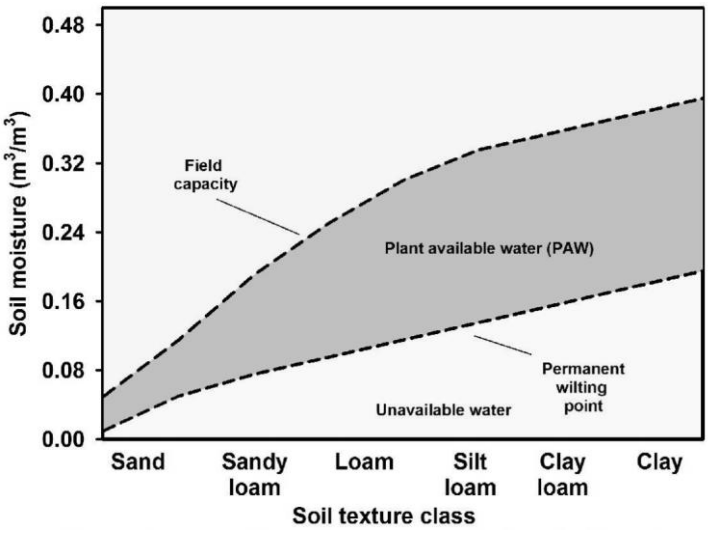


Figure 20. The relationship between plant available water (PAW), field capacity (or water holding capacity), unavailable water and permanent wilting point for soils of different texture classes (Zotarelli *et al.*, 2010).

The particle size of soil components plays an important role in determining the physical properties of soils. Soils containing fine particles are more moisture retentive than those with more coarse particles (Gil *et al.*, 2012; Ho and Zentmyer, 1977; Eden *et al.*, 2000). This is because there is stronger adhesion of water to fine particles than there is to more coarse particles (Evett *et al.*, 2008). The use of fine particles in avocado potting mix is not recommended as the micro-films of water that are created between fine soil particles provide environments where *Phytophthora* can breed (Gil *et al.*, 2012; Ho and Zentmyer, 1977; Eden *et al.*, 2000). Additionally, small particles in the soil decrease aeration which leads to compaction (Gil *et al.*, 2012; Ferreyra *et al.*, 2007). For growing avocados, it is recommended that soils should not contain particles that are finer than 0.5mm (Lee and Roxburgh, 1993). Over time, however, soils do weather and break down into smaller particles, with organic matter breaking down more quickly than sands (Wallach, 2007; Bunt, 2012). It is thus recommended that the medium for growing avocados in pots is stable in the long term, in that it does not break down rapidly and its structure is maintained.

3.1.4.2 Chemical characteristics

In terms of the chemical properties of soils, pH is an important factor in determining how well plants grow as it determines nutrient availability and uptake (Husson, 2013). While avocados have been grown on a variety of soil types, they grow best on soils which have a pH between 4.5 and 5.3 (Wolstenholme, 2013, 2011; Whiley and Schaffer, 1994).

Salinity affects avocado growth and development (Bernstein *et al.*, 2001). Salinity stress is mainly caused by dissolved chloride and sodium ions, which decrease the plants' ability to uptake water (Bernstein *et al.*, 2004). It has been observed that root growth is affected by salinity more than shoot growth in avocado (Bernstein *et al.*, 2004). The salinity of the soil can be determined using electrical conductivity (EC), which is measured in deciSiemens per meter (dS/m) (Crowley, 2008; Oster *et al.*, 2007; Gustafson, 1962). Soils with an EC reading (soil water) above 4 dS/m are too saline for the healthy growth and development of avocado plants (Crowley, 2008; Oster *et al.*, 2007; Gustafson, 1962).

3.1.5 Avocado soil mixes

To propagate avocados, nurseries have been using different growing media generally comprising of composted pine bark, peat moss, river sand and perlite, in different proportions (Ernst *et al.*, 2013). Organic matter in the medium is important as it contains the nutrients necessary for growth. Avocados have few to no root hairs, thus they have to form

associations with microorganisms that are in the soil in order to improve nutrient uptake (Lahav *et al.*, 2013; Burgis and Wolfe, 1945). Organic matter contains both nutrients and these microorganisms (Shepherd *et al.*, 2002; Nesbitt *et al.*, 1979; Broadbent and Baker, 1974). Additionally, these microorganisms have been observed to play a role in preventing *Phytophthora* root rot (Shepherd *et al.*, 2002; Nesbitt *et al.*, 1979; Broadbent and Baker, 1974).

Composted pine bark is currently used in avocado potting media as it is free-draining, it contains plant nutrients and has been found to be useful in supressing *Phytophthora* establishment (Wolstenholme *et al.*, 2013; Carlile, 2005; Odneal and Kaps, 1990). Pine bark, however, was observed to block the drainage holes at the bottom of potting bags, which then encouraged waterlogging (Bilderback, 1982). Peat (Canadian peat and coco peat) retains water while providing aeration, but does not have nutritional value (Landis and Morgan, 2009). Both perlite and (river) sand are used for increasing aeration in growing media (Landis and Morgan, 2009). Sand is extracted from river beds and perlite is made up of volcanic rock that has been expanded with heat. Sand has a high bulk density, while perlite has a low bulk density (Ernst, 1999).

In South Africa, a soil mix of 1:1:1 composted pine bark, sugar cane filter press, coarse river sand is mostly used for avocado propagation in nurseries (Ernst *et al.*, 2013). In other parts of the world, soil mixes that have been used include 1:5:1 perlite, peat moss, wetting agent, 2:1:1 sand, peat moss, nitrogenated red wood compost, 1:1 coarse river sand, peat and 1:1 river sand, pine bark (Ernst *et al.*, 2013). For conducting experiments, a range of soil types have been used to grow avocados in containers, ranging from sandy loam to soils rich in peat (Table 14).

Table 14. Soil types that have previously been described for the growth of avocado plants in containers for experimental purposes.

Variety	Plant age	Environment	Soil	Reference
'Choquette' grafted on 'Waldin' rootstocks	2 years	Shade house with 30% shade cloth	40% Canadian peat, 10% coir, 40% pine bark and 10% perlite	Sanclemente *et al.*, 2013
'Hass'	2 years	Greenhouse	Sand, lime, peat	Auger *et al.*, 2013
'Hass' grafted on 'Degania'	-	-	Perlite of 2-mm grain size with 50-L of coarse tuff (volcanic material)	Silber *et al.*, 2013, 2012

rootstocks			at the bottom	
'Hass' grafted on 'Mexicola' rootstocks	3 years	Outdoors	Heavy clay loam soil	Gil *et al.*, 2009
'Choquette' grafted on 'Waldin' rootstocks	1 year	-	50% Canadian peat, 40% pine bark and 10% sand by volume	Gil *et al.*, 2007
'Hass' grafted on 'Mexicola' rootstock	-	-	clay-loam soil	Cervera *et al.*, 2007
'Hass' grafted on 'Guatemalan' rootstock	3 years		2:1:1 pine bark, peat and river sand	Neuhaus *et al.*, 2007
'Fuerte'	3 months	Greenhouse	-	Ouma, 2007
West Indian rootstocks	1 years	Screenhouse	Peat and tuff	Bernstein *et al,*. 2001
'Hass' on grafted 'Duke 7' rootstock	2 years	-	Peat, perlite, compost and sand	Gil *et al.*, 2011
'Mexicola'	6 months old	-	100% compost	
'Hass' and 'Fuerte' grafted on Mexican rootstock	2 years	Outside	Free draining sandy loam	Chartzoulakis *et al.*, 2001
-	-	-	Sterile composted milled pine bark	Ernst, 1999
'Hass'	4-6 years	Outdoors	1:1 course river sand and peat	Whiley *et al.*, 1999
'Duke 7'	-	-	3:1:1 composted pine bark, sand, and peat	Mcleod *et al.*, 1995
'Edranol'	2 years	-	Soil-based nursery mix	Bower *et al.*,1977
'Mexicola'	7 weeks	Greenhouse	Fallbrook sandy loam	Stolzy *et al.*, 1967

3.1.6 Irrigation

Irrigation needs to be optimised for avocados in order to provide sufficient water for the plant to survive and thrive, without overwatering the plant which would cause root rot and other diseases (Salgado and Cautın, 2008; Salazar-Garcia and Cortes-Flores, 1986). In avocado

orchards, soil moisture meters are used to schedule irrigation (Salgado and Cautın, 2008; Salazar-Garcia and Cortes-Flores, 1986). To do this, soil water potential has been used to give an indication of how readily water is available from the soil to the plant and how much force is needed by the plant in order to use water from the soil (Lahav *et al.*, 2013; Silber *et al.*, 2012; Lahav and Kalmar, 1977). Soil moisture potential ranges from 0kpa to -85kpa, with waterlogged soils being at 0kpa and very dry soils at -85kpa (Lahav *et al.*, 2013; Silber *et al.*, 2012; Lahav and Kalmar, 1977). At -20kpa moderate force is needed to extract water from the soil, while at -60kpa and higher more force is required, and thus plants in these soil conditions will experience dehydration stress (Lahav *et al.*, 2013; Silber *et al.*, 2012; Lahav and Kalmar, 1977). Avocados are shallow rooted, with approximately 80% of feeder roots found in the top 25cm of soils (Lahav *et al.*, 2013; Salgado and Cautın, 2008; Du Plessis, 1991). In well-draining soils, it is possible for the top soil to dry out, which in turn would cause the plant dehydration stress (Salgado and Cautın, 2008; Salazar-Garcia and Cortes-Flores, 1986). In this regard, it was observed that avocados are sensitive to drying stress (Salgado and Cautın, 2008; Salazar-Garcia and Cortes-Flores, 1986).

Avocados require soils that are well-draining, aerated and rich in organic matter for optimum growth (Gil *et al.*, 2007). This is because avocado roots are sensitive to waterlogged environments, with the plants showing stress within 48 hours (Crowley, 2007; Schaffer *et al.*, 1999, 1992). Waterlogged environments are also characterised by low oxygen availability. This reduces stomatal conductance, the growth of roots and shoots, and promotes wilting and leaf drop (Schaffer *et al.*, 2013a; Gil *et al.*, 2009; Schaffer *et al.*, 1992). Additionally, waterlogged soils have a low pH and a temporary increase in nutrient availability which may be toxic for the roots of the plant (Schaffer *et al.*, 2013a; Gil *et al.*, 2009; Schaffer *et al.*, 1992).

It was suggested that irrigation for avocados should be applied when 30% of soil moisture has been depleted in coarse textured sandy soils, which corresponds to a soil moisture potential of about -30kpa (Lahav *et al.*, 2013; Zotarelli *et al.*, 2010). When irrigation is applied less frequently vegetative growth was observed to decrease (Silber *et al.*, 2012; Lahav and Kalmar, 1977). As a result, frequent irrigation (between 7 and 12 days) is required for rapid vegetative growth in avocado (Silber *et al.*, 2012; Lahav and Kalmar, 1977). However, with frequent irrigation in sandy soils nutrients are easily leached. Thus irrigation scheduling needs be optimised in order to supply water at an amount which is optimum for growth, while minimising the leaching of soil nutrients (Kiggundu *et al.*, 2012).

3.1.7 Vegetative growth in avocado

Growth of different tree species has been described and characterised using tree architecture models. Avocado follows the Rauh model of tree architecture, characterised by being monopodial (single trunk) with an upright growth (Alcaraz *et al.*, 2013; Mickelbart *et al.*, 2012). Avocado trees have two shoot types; namely, sylleptic shoots, which develop from non-dormant apical meristems, and proleptic shoots, which develop from apical meristems that have been dormant (Alcaraz *et al.*, 2013; Mickelbart *et al.*, 2012). Vegetative growth in avocado occurs in seasonal flushes in warmer climates (DAFF, 2012; Davenport, 1982).

3.1.8 Motivation

Based on what was observed in the initial experiments, where it was identified that the quality of the mother plants may have contributed to the low response of the explants *in vitro*, further research was conducted to develop a system to optimise stage 0 preparations, in order to generate healthy material to be used for the *in vitro* propagation of avocado. In the initial experiments, successful establishment of avocado mother plants was not achieved as many of the plants (more than 50%, data not shown) succumbed to *Phytophthora* root rot. Additionally, the plants that were not infected with root rot did not grow vigorously.

3.1.9 Aims and objectives

The aim of this study was to determine the best soil for growing healthy avocado plants (for subsequent use in micropropagation experiments) from two soil mixes, namely 1:1:1 pine bark, perlite, river sand and 1:1:1 peat, perlite, river sand.

The objectives of this study were to determine the:

- physical properties of the soils (water content, bulk density, water holding capacity, total porosity and air-filled porosity)
- chemical properties of the soil (pH, EC)
- physiological measurements of the plants after growing in the two soils

3.2 MATERIALS AND METHODS

3.2.1 Experimental design and protocols

3.2.1.1 Stage 0. Mother plant establishment and preparation

An experiment was conducted to determine the optimum growing medium for avocados in containers. This study was conducted in a greenhouse (26-28°C, 40% relative humidity) at the University of the Witwatersrand, Johannesburg (Figure 21.B). Seedlings (Figure 21.A) were obtained from Rietvlei Nursery and were planted in 20L plastic pots with drainage holes. To increase aeration and to prevent the pine bark from blocking the drainage holes, a single layer of gravel (13 mm) was placed at the bottom of each pot. The following growing media were used:

- 8mm composted pine bark
- Coco peat (Nutrigro)
- 3mm washed river sand
- Perlite (Granulite Groperl)

Two different avocado soil mixes were tested in the following ratios: 1:1:1 pine bark, perlite, river sand (referred to as the pine bark mix) and 1:1:1 peat, perlite, river sand (referred to as the peat mix). Eight seedlings were planted in each soil mix. For the prevention of root rot Ridomil Gold (0.5g/L of soil) was added into each pot. After planting, the seedlings were maintained under 60% shade cloth for two weeks and thereafter 40% shade cloth was used. The plants were drenched with 10L of water immediately after potting. After two weeks, the apical buds of the main stems (Figure 22) of the plants were excised to encourage axillary bud break. The plants were treated with the same chemical regime (fertilisers and fungicides) as described in Table 3 (Section 2.2.1.1), with the addition of monthly applications of Nitrosol (10ml per litre of water).

Figure 21. A. An avocado seedling that was used in this experiment. B. Avocado seedlings growing in a greenhouse, after being transplanted into plastic pots.

3.2.1.1.1 Irrigation

A moisture meter (HydroSense II) with a 20cm probe was used to monitor soil moisture and to determine when irrigation was needed. Irrigation was done by hand, and was applied frequently to maintain the soil mixture close to its water holding capacity.

3.2.1.1.2 Physical properties of the soil

The bulk density, volumetric water holding capacity and air-filled porosity were determined according to the methods of House (2011), Bilderback (1982) and Gessert (1976), and were replicated 3 times. A sample of the soil was filled into a 250ml (250 cm^3) plastic pot, then weighed. Both dry and wet bulk density (g/cm^3) was calculated as:

$$Bulk\ density = \frac{mass\ of\ soil}{volume\ of\ soil}$$

To calculate porosity, the holes at the bottom of the pot were sealed with tape and water was added until a thin film appeared above the soil surface, signifying soil saturation. The total porosity (%) of the soil was calculated as:

$$Total\ porosity = \frac{Pore\ volume}{Soil\ volume} \times 100$$

Where:

Pore volume: amount of water added to saturate soil, and fill pores (ml)

Soil volume: total soil and pore volume (ml)

To calculate the air-filled porosity, the tape was removed from the bottom of the pot and the water was left to drain freely from the pot. The volume of water that drained out was measured, and air-filled porosity (%) of the soil was measured as:

$$Air\ filled\ porosity = \frac{Volume\ drained}{Soil\ volume} \times 100$$

Where:
Volume drained: amount of water drained from soil after saturation

The water holding capacity (%) of the soil was calculated as:

$$Water\ holding\ capacity\ = total\ porosity - air\ filled\ porosity$$

3.2.1.1.3 Chemical soil properties

The soil pH and EC (10g of soil sample in 20ml distilled water) were measured at 23°C, using a Thermo Scientific Orion Meter. Two soil samples were taken per pot (8 pots were used in total).

3.2.1.1.4 Physiological measurements

Eleven weeks after transplanting the seedlings, the following measurements of growth and vigour were taken from the plants growing on both soil mixes:

- The number of primary shoots (Figure 22)
- Length of primary shoots (cm)
- The number of buds per primary shoot

After 11 weeks, the apical buds of primary shoots were removed, to promote axillary bud break. Five months after potting the seedlings, the following was measured:

- The number of secondary shoots (Figure 22)
- The number of buds per secondary shoot

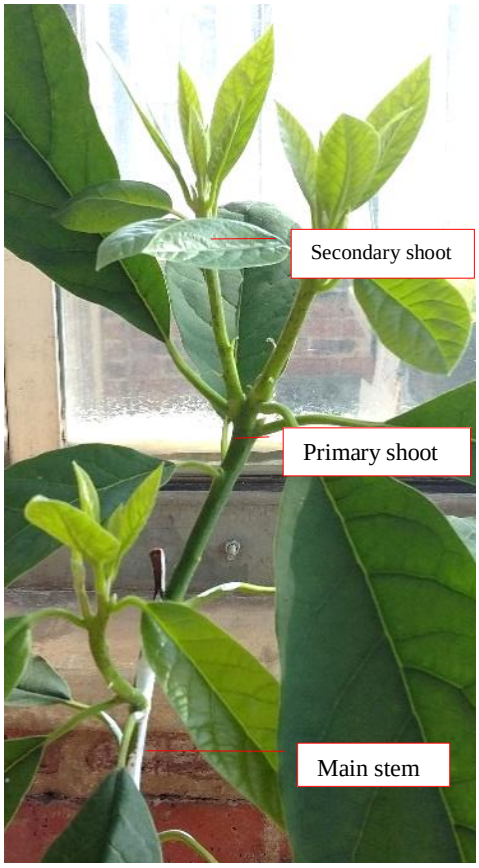


Figure 22. An avocado seedling, with the main stem, primary shoots and secondary shoots.

3.2.2 Data analysis

The physical and chemical properties of the two soils, and the physiological measurements of the plants growing on the two soils were analysed using Students t-tests. All statistical tests were conducted at *0.05* significance level and were done with the program STATISTICA

3.3 RESULTS

In terms of the physical properties of the two soils, the water holding capacity of the peat mix (33.4 ± 2.45%) was (significantly) higher than that of the pine bark mix (23.33 ± 2.40%), while the air-filled porosity was higher in the pine bark mix (26.8 ± 2.62%) compared with the peat mix (16.8 ± 0.65%) (Table 15). There was no difference in the total porosity and the dry bulk density between the two soil mixes (Table 15). The wet bulk density of the peat mix (1.42 ±0.05), however, was higher than that of the pine bark mix (1.15 ±0.04) (Table 15).

In terms of the chemical properties of the two soils, there was no difference in the soil pH, with pH ranging between 6.24 ± 0.06 and 6.59 ± 0.04 (Table 15) for each of the soil mixes, respectively. EC, however, was significantly higher in the peat mix (0.44 ± 0.07 dS/m) than in the pine mix (0.20 ± 0.01 dS/m).

Table 15. Physical and chemical properties of the two soil mixes tested in this study, namely 1:1:1 pine bark, perlite, river sand and 1:1:1 peat, perlite, river sand.

	1:1:1 Pine bark, perlite, river sand	1:1:1 Peat, perlite, river sand	*p value*
Physical measurements			
Water holding capacity (%)	23.33 ± 2.40	33.40 ± 2.45	*0.042*
Dry bulk density (g/cm^3)	0.86 ± 0.01	0.86 ± 0.04	*0.940*
Wet bulk density (g/cm^3)	1.15 ±0.04	1.42 ±0.05	*0.023*
Total porosity	50.4 ± 2.22	50.6 ± 1.76	*0.929*
Air-filled Porosity	26.8 ± 2.62	16.8 ± 0.65	*0.025*
Chemical measurements			
pH	6.24 ± 0.06	6.59 ± 0.04	*0.259*
EC (dS/m)	0.20 ± 0.01	0.44 ± 0.07	*0.003*

After 11 weeks of growth in the two soil mixes, there were no differences in any of the physiological measurements taken from the avocado seedlings (Table 16). For both soil mixes, the number of primary shoots ranged between 2.0 ± 0.26 and 2.2 ± 0.27, while the number of axillary buds per shoot ranged between 5.5 ± 0.56 and 6.1 ± 0.55 for each soil type, respectively (Table 16). Shoot length was observed to range between 7.3 ± 1.29 cm and 8.77 ± 1.39 cm (Table 16).

Table 16. Physiological measurements (The number (mean ± SE) of primary shoots, axillary buds per shoot and shoot length (cm)) of avocado seedlings, after 11 weeks of growth on two soil mixes (1:1:1 pine bark, perlite, river sand and 1:1:1 peat, perlite, river sand).

	1:1:1 Pine bark, perlite, river sand	1:1:1 Peat, perlite, river sand	*p value*
Physiological measurements			
Number of primary shoots	2.0 ± 0.26	2.20 ± 0.27	*0.575*
Number of axillary buds per primary shoot	5.5 ± 0.56	6.10 ± 0.55	*0.458*
Shoot length (cm)	7.30 ± 1.29	8.77 ± 1.39	*0.552*

After 5 months, there was no significant difference in the number of secondary shoots and the number of axillary buds per secondary shoot of avocado seedlings grown in the two soil types (Table 17). The number of secondary shoots ranged between 2.9 ± 0.31 and 3.37 ± 0.32 on

the pine bark mix and the peat mix respectively, while the number of axillary buds per secondary shoot was observed to be 7.75 ± 0.39 and 6.33 ± 0.53 (Table 17).

Table 17. Physiological measurements (The number (mean ± SE) of secondary shoots and axillary buds per secondary shoot) of avocado seedlings, after 5 months of growth in two soil mixes (1:1:1 pine bark, perlite, river sand and 1:1:1 peat, perlite, river sand).

	1:1:1 Pine bark, perlite, river sand	1:1:1 Peat, perlite, river sand	*p value*
Physiological measurements			
Number of secondary shoots	2.90 ± 0.31	3.37 ± 0.32	*0.312*
Number of axillary buds per secondary shoot	7.75 ± 0.39	6.33 ± 0.53	*0.123*

3.4 DISCUSSION

3.4.1 Stage 0. Mother plant establishment and preparation

In the initial experiments, it was hypothesised that the soil characteristics contributed to the avocado plants developing root rot. Thus, a system was developed to establish the plants. The physical properties of the two soils tested were similar, as pine bark and peat made up only a third of each potting mix respectively. Pine bark and peat have different properties. Peat has finer particles, with lower air filled porosity, and is more moisture retentive, with a higher water holding capacity (33.4 ± 2.45%) than pine bark (23.33 ± 2.40%) (Wallach, 2007; Gessert, 1976). It is for this reason that peat is heavier than pine bark when wet, thus has a higher wet bulk density (Heiskanen, 1996). The soil pH was similar for both soil types (6.24 and 6.59) and at a non-limiting level for growth in avocado plants (Wolstenholme, 2013; Gil *et al.*, 2012). While soil EC was different (Table 15), for both soil mixes it was at an acceptable level, i.e. it was below 4 dS/m, which is the level which imposes salinity stress on avocado plants (Crowley, 2008; Oster *et al.*, 2007; Gustafson, 1962).

The pine bark mix had physical properties similar to a sandy loam, while the peat mix was similar to loam soil (Claassens and Welgemoed, 2011; Bilderback, 1982; USDA, n.d.). Both of these soil mixes provided conditions favourable for growing avocados, namely organic matter, high aeration, low bulk density and were well-draining yet moist (Gil *et al.*, 2012; Gil, *et al.*, 2009). While some differences were observed in the bulk density, field capacity and EC of the two soil mixes tested (Table 15), these differences did not appear to affect the growth of the seedlings after 11 weeks and after 5 months (Table 16 and 17). The seedlings

produced a sufficient number of primary shoots and secondary shoots (i.e. more than 2 shoots), with axillary buds, to perform further *in vitro* experiments. The primary shoots were well extended (7.3 ± 1.29cm; 8.7 ± 1.39cm) (Table 16). The plant growth and development that was observed on both soil mixes was typical of that observed in other avocado plants (Alcaraz *et al.*, 2013). Thus, it was concluded that that either the pine bark (1:1:1 pine bark, perlite, river sand) or the peat based soil mix (1:1:1 peat, perlite, river sand) can be used to establish avocado mother plant seedlings in containers, for the purpose of generating vegetative shoots for avocado tissue culture.

3.4.2 Other culture conditions

While soil type was identified as the major cause of root rot and plant deterioration, other conditions were improved in this study. Potting soil was used in the initial experiments. Commercial potting mixes contain a mixture of bark and peat, and other aggregates (Claassens and Welgemoed, 2011; Landis and Morgan, 2009; Miller and Jones, 1995). These potting mixes are not graded, and the finer particles tend to contribute to soil waterlogging (Claassens and Welgemoed, 2011; Landis and Morgan, 2009; Miller and Jones, 1995). Additionally, potting soils and those high in organic matter break down rapidly, which increases soil compaction and decreases soil aeration and drainage. These conditions favour the development of *Phytophthora* root rot. Placing a layer of coarse gravel at the bottom of the pots prevented the drainage holes from getting blocked with the pine bark. The use of containers (pots) to grow avocados for experimental purposes was more suitable than planting bags, as pots provided more stability for both the plant and the soil.

3.4.3 Recommendations

Based on the findings of this study in order to establish healthy avocado mother plants, the following recommendations are made:

- A well-draining, coarse textured soil with organic matter (either peat or pine bark) should be used to cultivate avocado mother plants
- Plants should be grown in plastic pots, with a layer of gravel placed at the bottom

4 CHAPTER FOUR - Establishment of a System for The Proliferation of *In Vitro* Avocado Axillary Buds

4.1 INTRODUCTION

4.1.1 Further development of the tissue culture system

Based on the recommendations of the initial experiments (Chapter Two) further experiments were conducted to optimise the system for the *in vitro* propagation of avocado. The findings suggested that 1mg/l BAP and either ½ MS or 1MS could be used to initiate avocado cultures, but recommended that a concentration higher than 1mg/l BAP would be needed for explant multiplication. Additionally it appeared that ½ MS promoted hyperhydricity at subsequent subcultures, and this was attributed more to the concentration of micronutrients and vitamins in the medium than the concentration of macronutrients (in particular nitrogen). Based on those observations the *Persea indica* medium as proposed by Nel *et al.* (1983) was considered. Those authors noted some success using this medium to propagate avocados, but no data was given regarding the actual growth of 'Edranol' propagules. The composition of the MS based medium used in the previous experimentation and the *Persea indica* medium (Nel *et al.*, 1983) are compared in Table 18.

Table 18. The concentration (mg/l) of the components (macronutrient, micronutrients, vitamins, plant growth regulators, carbon source, gelling agents and antioxidants) in MS medium at full strength (Murashige and Skoog, 1962) (as was used in Chapter Two) and in the *P. indica* medium (Nel *et al.*, 1983).

	MS full strength	*P. indica*
Macronutrients	332.02 $CaCl_2$ 170 KH_2PO_4 1900 KNO_3 180.54 $MgSO_4$ 1650 NH_4NO_3	166.01 $CaCl_2$ 85 KH_2PO_4 950 KNO_3 90.27 $MgSO_4$ 825 NH_4NO_3 170 $NaH_2PO_4.2H_2O$
Micronutrients	0.025 $CoCl_2.6H_2O$ 0.025 $CuSO_4.5H2O$ 36.70 FeNaEDTA 6.20 H_3BO_3 0.83 KI 16.90 $MnSO_4.H_2O$ 0.25 $Na_2MoO_4.2H_2O$ 8.60 $ZnSO_4.7H_2O$	0.025 $CoCl_2.6H_2O$ 0.025 $CuSO_4.5H_2O$ 25 FeNaEDTA 6.20 H_3BO_3 0.83 KI 16.90 $MnSO_4.H2O$ 0.25 $Na_2MoO_4.2H_2O$ 8.60 $ZnSO_4.7H2O$
Vitamins, amino acids	2 Glycine 100 myo-Inositol 0.50 Nicotinic acid 0.50 Pyridoxine HCl 0.10 Thiamine HCl	2 Glycine 100 myo-Inositol 1 Nicotinic acid 1 Pyridoxine HCl 1 Thiamine HCl

		80 Adenine sulphate 1 Pantothenic acid (Ca-salt)
Plant growth regulators	1 BAP	2 BAP 1 GA_3
Carbon source and gelling agent	30 sucrose 3 Gelrite	30 sucrose (liquid medium – no gelling agent)
Antioxidant		25mg/l ascorbic acid

4.1.1.1 Nutrients

The nutrients that have been observed to be most important for plant growth are categorised as macronutrients and micronutrients (George and De Klerk, 2008). These categories are based on the amounts in which the nutrients are needed rather than their importance (Razdan, 2003). For instance iron, a micronutrient, is of utmost importance for growth and development in plants as it plays a role in the biosynthesis of chlorophyll but is only required in small amounts for normal plant functioning (Minocha and Jain, 2000; Wessels, 1996; Harty, 1985). The macronutrients which have been used in MS medium are nitrogen (N), potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg) and sulphur (S). Monosodium phosphate dihydrate ($NaH_2PO_4.2H_2O$) functions as an additional source of phosphorus and has been used both in avocado tissue culture (Schall, 1987; Nel *et al.*, 1983) and in other tissue culture formulations such as Gamborg's medium (Gamborg *et al.*, 1968) and White's medium (White, 1963).

The micronutrients that have been used are copper (Cu), chorine (Cl), iron (Fe), boron (B), manganese (Mn), molybdenum (Mo), zinc (Zn), iodine (I), sodium (Na) and cobalt (Co) (George and De Klerk, 2008; Trigiano and Gray, 1999). The micronutrients were used in the full strength MS concentration in the *P. indica* medium (Nel *et al.*, 1983), except for iron, in the form of FeNaEDTA (Ethylenediaminetetraacetic acid ferric sodium salt), which was reduced from 36.7mg/l to 25 mg/l. This is the same concentration used in Woody Plant Medium (McCown and Lloyd, 1981). For avocado tissue culture some researchers have used FeNaEDTA at 25mg/l (Schall, 1987; Nel and Kotze, 1982) while others have used it in the MS standard concentration (Rohim *et al.*, 2013; Zulfiqar *et al.*, 2009; Nhut *et al.*, 2008).

Vitamins are produced in plants in small quantities (Thorp *et al.*, 2008; Razdan, 2003). In the tissue culture of some species the addition of vitamins has been observed to improve initial growth significantly, while in other species it has had no effect on growth (Thorp *et al.*, 2008; Razdan, 2003). The commonly used vitamins in plant tissue culture belong to the vitamin B

group (Thorp *et al.*, 2008; Razdan, 2003). Because the benefit of adding vitamins ranges from being very significant to having no effect at all, experiments need to be carried out to determine which vitamins are needed and in what concentrations (Thorp *et al.*, 2008; Razdan, 2003).

The presently used MS formulation contained the following vitamins: 0.5 mg/l pyridoxine HCl (Vitamin B6), 0.1mg/l thiamine HCl (Vitamin B1), 0.5 mg/l nicotinic acid (Vitamin B3) and myo-inositol (Vitamin B complex) (Murashige and Skoog, 1962). In contrast to MS, double the concentration of pyridoxine HCl and nicotinic acid was used in the *P. indica* medium (Nel *et al.*, 1983). For avocado tissue culture Schaffer *et al.* (1999) and Nel *et al.* (1983) used 1mg/l thiamine while many other authors have used 0.4 mg/l (Litz and Witjaksono, 1999; Castro *et al.*, 1995; Zirari and Lionakis, 1994; Vega, 1989; Fernando Pliego-Alfaro and Murashige, 1988).

Other additions to plant tissue culture media include pantothenic acid (Ca-salt), which has been observed to promote growth in certain species, while having no effect on others (Thorp *et al.*, 2008). For avocado tissue culture it was used by Nel *et al.* (1983), and Nel and Kotzé (1982). Adenine sulphate behaves similar to a cytokinin, however it was observed to have inhibitory effects in some species (Van Staden *et al.*, 2008). While some researchers have included adenine sulphate in avocado tissue culture, it has not been used widely (Barrera-Guerra *et al.*, 2001; Nel and Kotze, 1982).

4.1.1.2 Plant growth regulators- gibberellins

The most common plant growth regulators used to induce *in vitro* axillary bud multiplication are the auxins, cytokinins and gibberellins. For avocado micropropagation studies, the use of auxin has been limited as the general responses of the explants suggest that explants produce high levels of endogenous auxin (Zulfiqar *et al.*, 2009; Nhut *et al.*, 2008). The cytokinin BAP has been widely used at varying concentrations. 2mg/l BAP was used by Barrera-Guerra *et al.* (2001), Vega (1989) and Nel and Kotzé (1982) for avocado tissue culture. Gibberellic acid (GA_3) plays a role in shoot growth through the elongation of internodes (Moshkov *et al.*, 2008). The use of 1mg/l GA_3 was suggested by Sánchez-Romero *et al.* (2007), Young (1983), Vega (1989) and Nel *et al.* (1983).

4.1.1.3 Gelling agents

Nel *et al.* (1983) used the *P. indica* medium in liquid form with filter paper bridges to support avocado nodal explants. Based on experiments conducted in this study (Chapter Two), it was decided that a semi-solid medium was more suitable than a liquid medium. This is because liquid media have been used for explants that display apical necrosis (Bairu *et al.*, 2009; Kataeva *et al.*, 1991), which was not observed in cv. 'Edranol'. Furthermore, hyperhydricity, which was observed in this study on semi-solid media (Chapter Two), would be enhanced with a liquid medium and this would be undesirable for *in vitro* production.

4.1.1.4 Antioxidants

The use of antioxidants was suggested for cv. 'Edranol', as tissue browning was observed (Chapter Two). In this regard, ascorbic acid was used as an antioxidant for avocado tissue culture, as recommended by Castro *et al.* (1995) and Nel *et al.* (1983).

4.1.2 Rationale/ motivation

The *P. indica* medium (Nel *et al.* 1983) was considered for the multiplication of *in vitro* avocado axillary buds as it contained many of the recommendations from Chapter Two. This medium, however, included other supplements, such as vitamins and antioxidants. To determine the optimum concentration of the components needed in plant tissue culture media experimental testing is needed. This is important both for optimal plant growth and for economic reasons; if certain supplements have no benefit for *in vitro* productivity, it is more economical not to add them.

4.1.3 Aims

The aim of this study was to further improve the *in vitro* system for the proliferation and multiplication of axillary buds in avocado (*P. americana*) cv. 'Edranol' by better understanding the responses of the explants to the combination of supplements in the tissue culture medium.

The objectives of this study were:

- to determine the physiological responses of *in vitro* avocado explants to two tissue culture media based on the *P. indica* medium (namely, P.I 1 and P.I 2)
- to compare the physiological responses of the explants on these two media with ½ MS + 1mg/l BAP and 1MS + 1mg/l BAP

4.2 MATERIAL AND METHODS

4.2.1 Experimental design and protocols

4.2.1.1 Stage 1. Establishment of an aseptic culture

Nodal explants from healthy avocado mother plants were excised from plants grown in both soil mixes mentioned in Chapter Three. The same surface decontamination protocol was used as described in Section 2.2.1.2.

4.2.1.2 Stage 2. In vitro establishment and multiplication

After surface decontamination, 1-2 explants were plated on 50ml semi-solid medium, in Magenta jars. Based on the *P. indica* medium (Nel *et al.*, 1983), two tissue culture media, named as P.I 1 and P.I 2, were tested as detailed in Table 19.

Table 19. Composition of P.I 1 and P.I 2 media.

P.I 1	P.I 2
½ MS Macro-elements	½ MS Macro-elements
Full MS Microelements	Full MS Microelements modified with 25mg/l FeNaEDTA
30mg/l sucrose	30mg/l sucrose
25mg/l ascorbic acid	25mg/l ascorbic acid
100mg/l *myo*-inositol	100mg/l *myo*-inositol
2mg/l glycine	2mg/l glycine
1mg/l pyridoxine HCl	1mg/l pyridoxine HCl
0.4 mg/l thiamine HCl	1mg/l thiamine HCl
1mg/l nicotinic acid	1mg/l nicotinic acid
2mg/ l BAP	2mg/ l BAP
1mg/l GA	1mg/l GA
3g/l Gelrite	3g/l Gelrite
	170mg/l $NaH_2PO_4.2H_2O$
	1mg/l pantothenic acid (Ca-salt)

All media were at pH 5.6 to 5.8. The explants were initially cultured in the dark for 7-10 days at 25°C and thereafter transferred to a 16-hour photoperiod. After 6 weeks, measurements of vigour and viability, as described in Section 2.2.1.4, were recorded.

4.2.1.3 Subculture

Explants were subcultured after 6 weeks as described in Section 2.2.1.5.

4.2.2 Sample numbers

Each experiment was replicated three times, with 8-10 samples per replicate.

4.2.3 Data analysis

The data was analysed as described in Section 2.2.2. Note: The responses of the explants that were cultured on ½ MS + 1mg/l BAP and 1MS + 1mg/l BAP (Chapter Two), have been presented again to ease comparison.

4.3 RESULTS

4.3.1 Stage 1. Establishment of an aseptic culture

Six weeks after *in vitro* establishment, there was a lower percentage contamination of explants cultured on the two P.I media (4.87% and 0%) than in the initial experiments (26.6% and 23%) (Figure 23.A). At the first subculture however, there was no difference in the percentage contamination of explants (Figure 23.B).

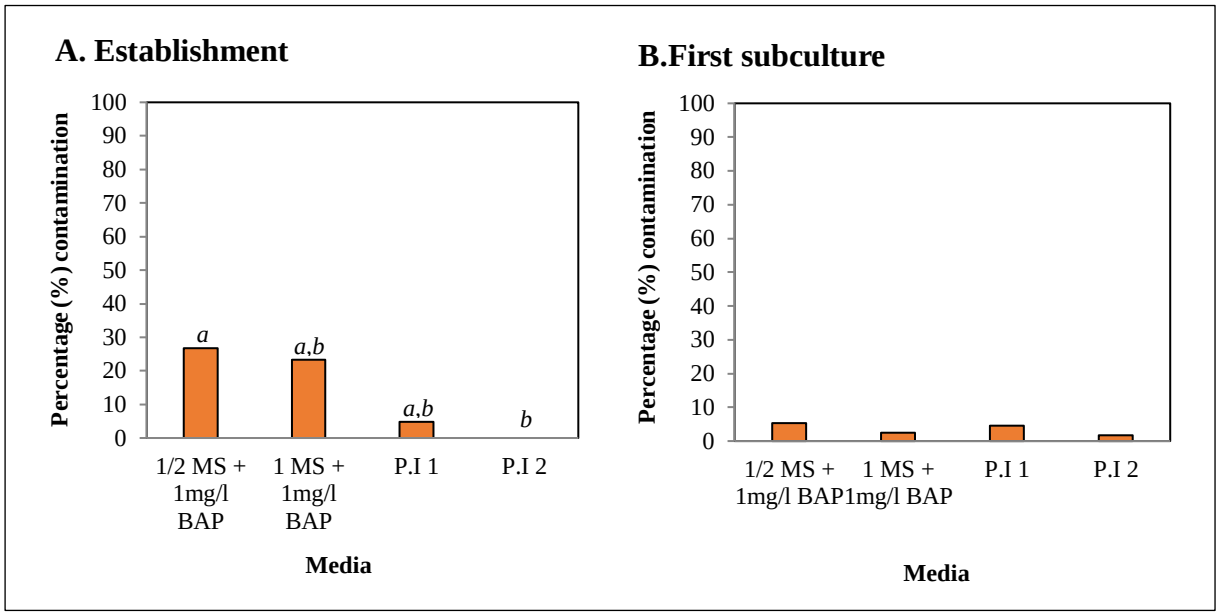


Figure 23. Percentage contamination of *in vitro* avocado axillary bud explants cultured on four tissue culture media within 6 weeks of establishment (A) and the first subculture (B). There was a significant difference in the percentage contamination at establishment (*p=0.01*), while there was no difference at the first subculture (*p=0.76*).

There was no difference in the percentage contamination of explants for both P.I media from establishment to the first subculture (Table 20).

Table 20. Percentage (%) contamination of *in vitro* avocado axillary bud explants cultured on two tissue culture media within 6 weeks of establishment and the first subculture.

	Percentage (%) contamination		
Medium	Establishment	First subculture	*p* values
P.I 1	4.87	4.47	*p=0.96*
P.I 2	0	1.69	*p>0.05*

4.3.2 Stage 2. *In vitro* establishment and multiplication

After establishment 87% of explants cultured on P.I 1 and 64% of those cultured on P.I 2 had pink, light green leaves (Figure 24.A), instead of dark green leaves as was observed in the initial experiments (data not shown) (Figure 24.B).

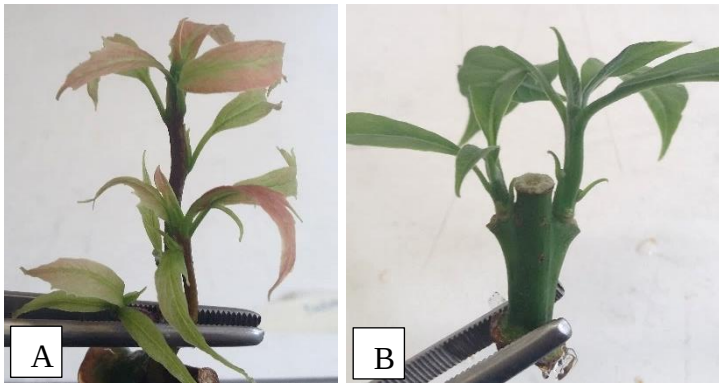


Figure 24. *In vitro* avocado explants with A. pink, light green leaves and B. dark green leaves.

4.3.2.1 Tissue browning

After six weeks of establishment, the highest tissue browning score was observed for explants cultured on P.I 2 (1.16 ± 0.06) (Figure 25.A). Tissue browning, however, did not exceed score 2 on all the media tested (Figure 25.A). Six weeks after the first subculture the highest scores for tissue browning was observed with explants cultured on 1MS + 1mg/l BAP (2.71 ± 0.30) (Figure 25.B).

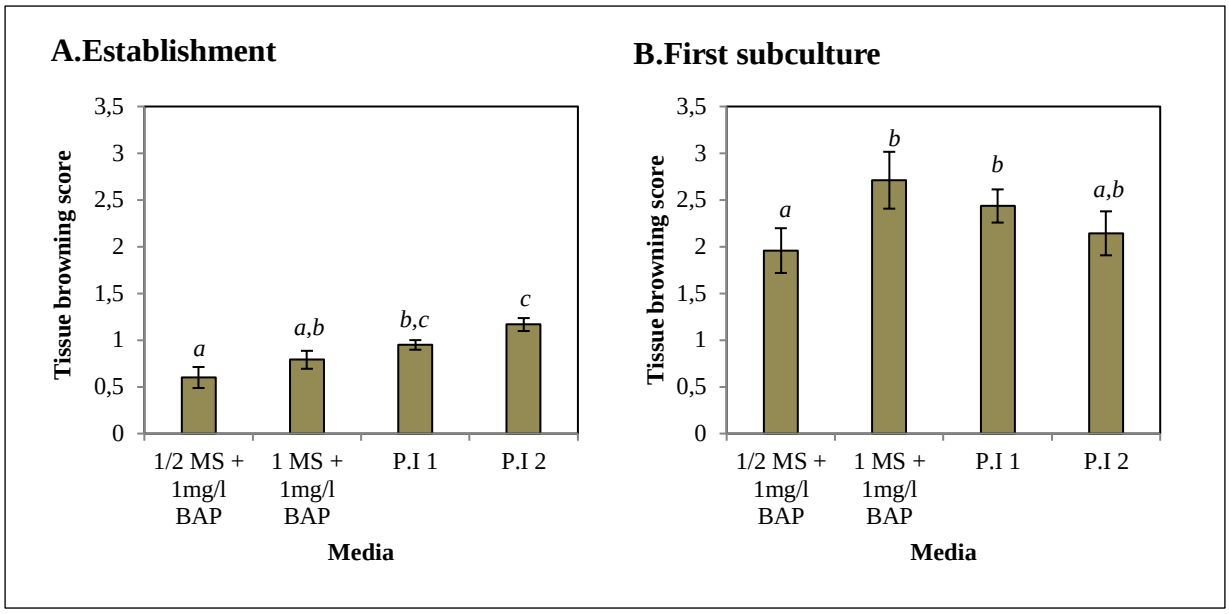


Figure 25. Tissue browning scores (mean ± SE) of *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment (A) and the first subculture (B). There was a significant difference in the tissue browning scores at establishment (*H(3, 108) =22.38, p<0.0001*) and at the first subculture (*H(3,236)= 12.15, p=0.007*).

There was an increase in the tissue browning scores for explants cultured on both P.I media from establishment to the first subculture (Table 21).

Table 21. Tissue browning scores (mean ± SE) of *in vitro* avocado axillary bud explants cultured on two tissue culture media 6 weeks after establishment and the first subculture.

	Tissue browning scores		
Medium	Establishment	First subculture	*p* values
P.I 1	0.94 ± 0.05	2.43 ± 0.06	*p< 0.0001*
P.I 2	1.16 ± 0.17	2.14 ± 0.23	*p=0.01*

4.3.2.2 Percentage death

There was no difference in the percentage death of explants between all the media tested after establishment (Figure 26.A). After the first subculture however the highest percentage of death (25.79%) was observed with explants cultured on 1MS + 1mg/l BAP (Figure 26.B).

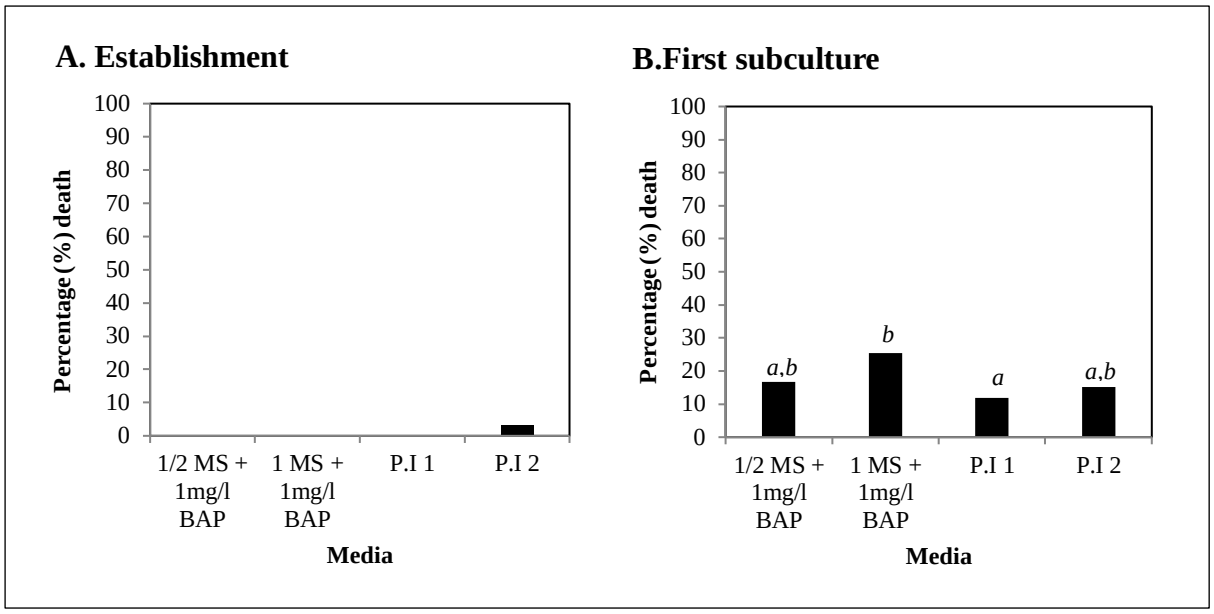


Figure 26. The percentage (%) death of *in vitro* avocado axillary bud explants cultured on four tissue culture media within 6 weeks of establishment (A) and the first subculture (B). There was no difference in the percentage death of explants at establishment (*p>0.05*), however there was a difference at the first subculture (*p=0.01*).

While the percentage death of explants increased numerically from establishment to the first subculture, it was not significantly different for both P.I media (Table 22).

Table 22. The percentage (%) death of *in vitro* avocado axillary bud explants cultured on two tissue culture media within 6 weeks of establishment and the first subculture.

	Percentage (%) death		
Medium	Establishment	First subculture	*p* values
P.I 1	0	11.94	*p=0.44*
P.I 2	3.12	15.25	*p=0.22*

4.3.2.3 Hyperhydricity

Hyperhydricity was higher on the two P.I media than in the initial experiments during establishment (17.94% and 10% respectively) and the first subculture (53.10%; 41.75%) (Figure 27).

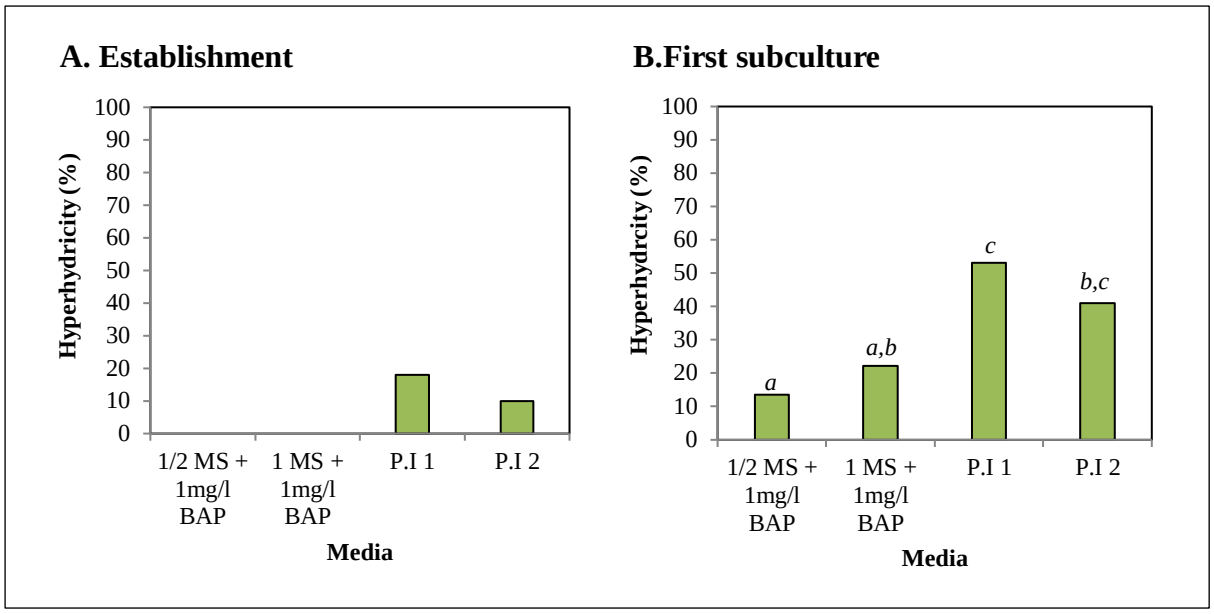


Figure 27. The percentage (%) hyperhydricity observed in *in vitro* avocado axillary bud explants cultured on four tissue culture media after 6 weeks of establishment (A) and the first subculture (B). There was no difference in the percentage hyperhydricity at establishment (*p=0.048*), however, there was a difference at the first subculture (*p< 0.0001*).

There was an increase in the percentage hyperhydricity for both P.I media tested from establishment to the first subculture (Table 23).

Table 23.The percentage (%) hyperhydricity observed in *in vitro* avocado axillary bud explants cultured on two tissue culture media within 6 weeks of establishment and the first subculture.

	Percentage (%) hyperhydricity		
Medium	Establishment	First subculture	*p* values
P.I 1	17.94	53.12	*p< 0.0001*
P.I 2	10	41.07	*p< 0.0001*

4.3.2.4 Number of shoots

The number of shoots that developed was less than 2 per explant on all the media tested (Figure 28). There was a significantly higher number of shoots that developed from explants cultured on P.I 1 (1.53 ± 0.15) than the three other media tested after establishment (Figure 28.A). After the first subculture, a higher number of shoots were developed from explants cultured on both P.I media than the initial media (Figure 28.B).

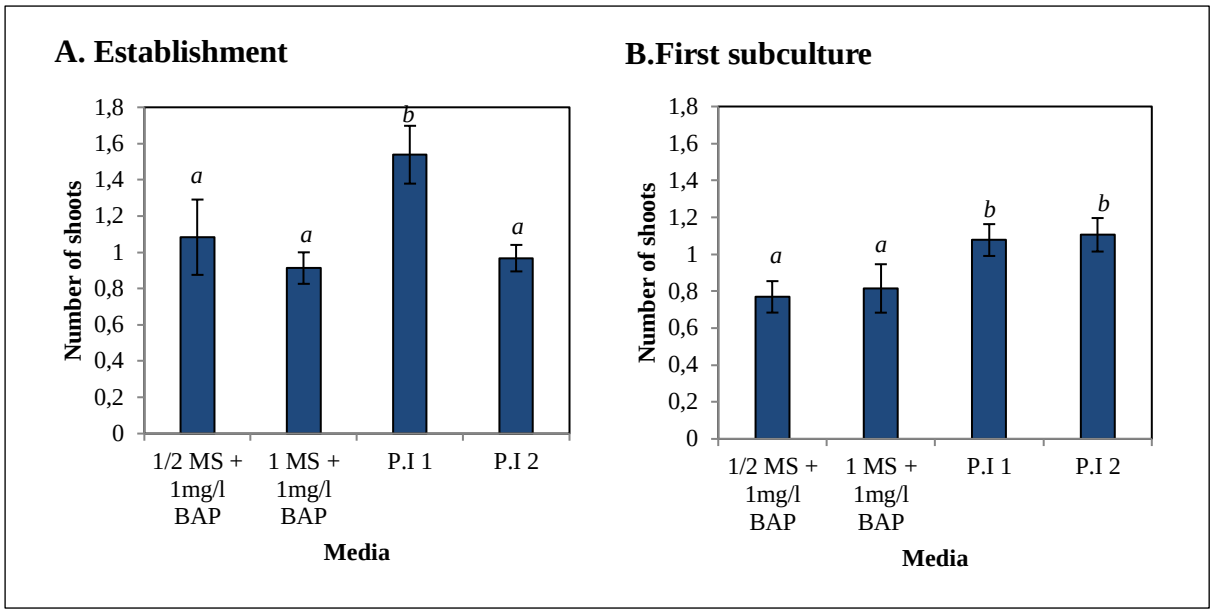


Figure 28. The number of shoots (mean ± SE) that developed from *in vitro* avocado axillary bud explants cultured on four tissue culture media after 6 weeks of establishment (A) and the first subculture (B). There was a significant difference in the number of shoots developed at establishment (*H(3,177)= 10.83, p=0.013*) and at the first subculture (*H(3,177)= 12.98, p=0.005).*

The number of shoots decreased for explants cultured on P.I 1 from establishment to the first subculture, but remained the same for those cultured on P.I 2 (Table 24).

Table 24. The number of shoots (mean ± SE) that developed from *in vitro* avocado axillary bud explants cultured on two tissue culture media 6 weeks after establishment and the first subculture.

	Number of shoots (mean ± SE)		
Medium	Establishment	First subculture	*p* values
P.I 1	1.53 ± 0.15	1.07 ± 0.08	*p=0.03*
P.I 2	0.96 ± 0.07	1.10 ± 0.09	*p=0.3*

4.3.2.5 Number of axillary buds

After establishment explants cultured on P.I 1 produced the highest number of axillary buds (7.17 ± 0.49) when compared with the three other media tested (Figure 29.A). However, there was no difference in the number of axillary buds developed after the first subculture on all four media tested (Figure 29.B).

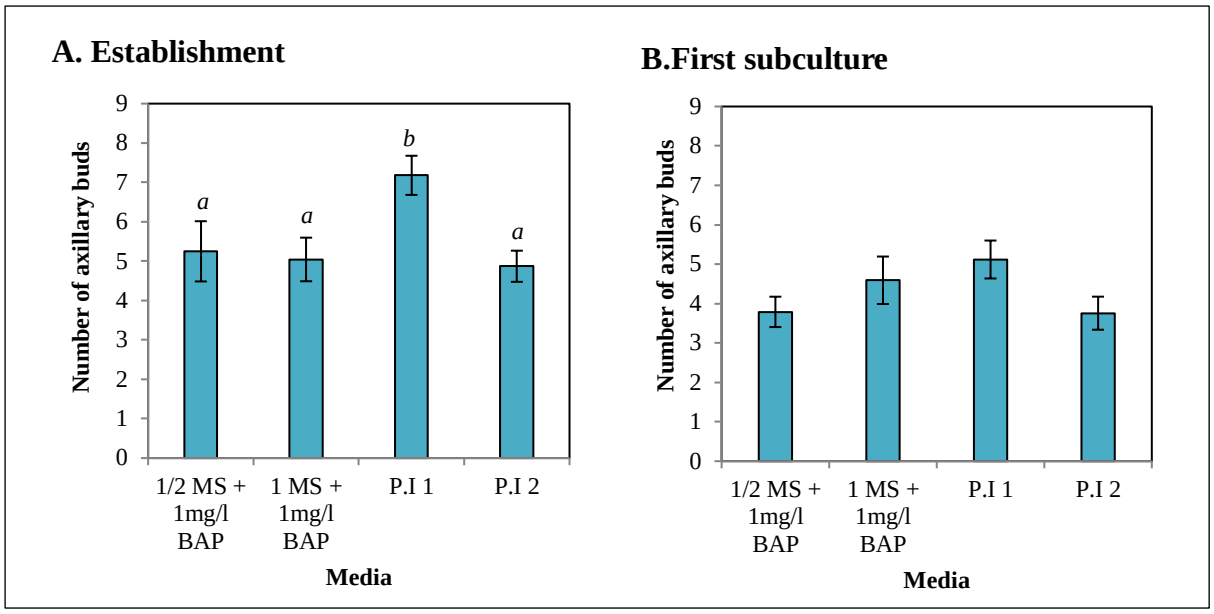


Figure 29. The number of axillary buds (mean ± SE) that developed from *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment (A) and the first subculture (B). There was a significant difference in the number of axillary buds that developed at establishment (*H(3,177)= 11.42, p=0.01*). However, there was no significant difference at the first subculture (*H(3,178)= 6.11, p=0.106*).

There was a significant decrease in the number of axillary buds which developed from both P.I media from establishment to the first subculture (Table 25).

Table 25.The number of axillary buds (mean ± SE) that developed from *in vitro* avocado axillary bud explants cultured on two tissue culture media 6 weeks after establishment and the first subculture.

	Number of axillary buds (mean ± SE)		
Medium	Establishment	First subculture	*p* values
P.I 1	7.17 ± 0.49	5.11 ± 0.48	*p=0.001*
P.I 2	4.87 ± 0.39	3.75 ± 0.41	*p=0.01*

4.3.2.6 Shoot length

Explants cultured on both P.I media produced shorter shoots than those cultured on the media tested in the initial experiments after establishment and the first subculture (Figure 30). The tallest shoots were produced from explants culture on 1MS + 1mg/l BAP six weeks after establishment and the first subculture (Figure 30).

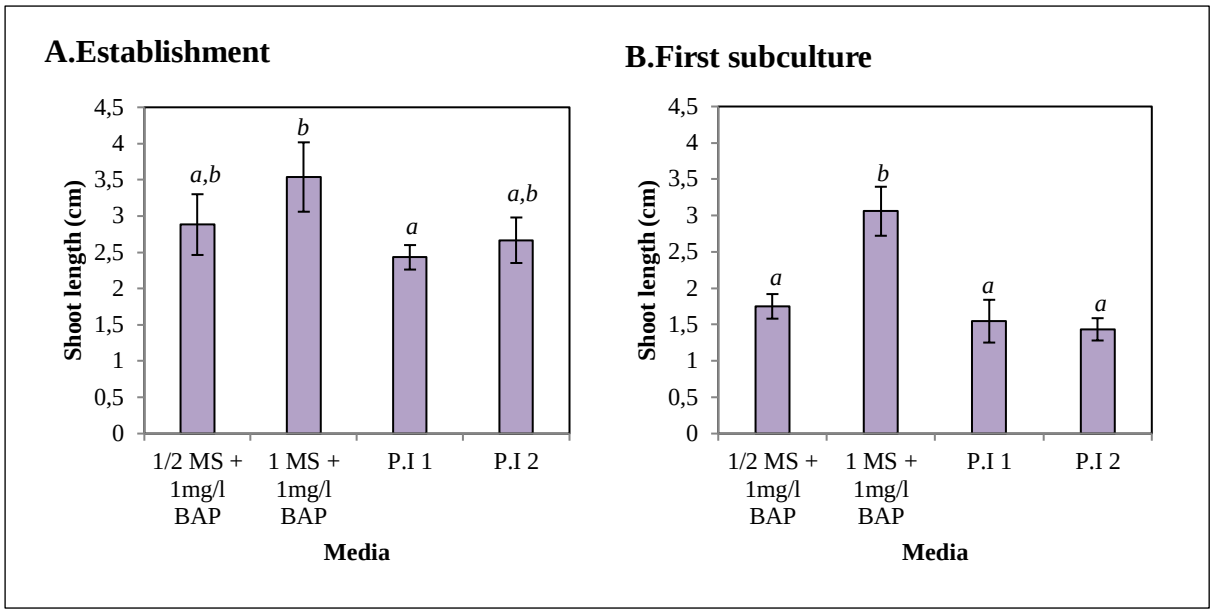


Figure 30. Shoot length (mean ±SE) (cm) of *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment (A) and the first subculture (B). There was a significant difference in shoot length at establishment (*H(3,64)= 10.85, p=0.01*) and at the first subculture (*H(3,134)= 27.17, p< 0.0001*).

There was a significant decrease in the length of shoots developed from explants cultured on both P.I media from establishment to the first subculture (Table 26).

Table 26. Shoot length (mean ±SE) (cm) of *in vitro* avocado axillary bud explants cultured on two tissue culture media 6 weeks after establishment and the first subculture.

	Shoot length (mean ±SE) (cm)		
Medium	Establishment	First subculture	*p* values
P.I 1	2.43 ± 0.16	1.54 ± 0.29	*p< 0,0001*
P.I 2	2.66 ± 0.31	1.43 ± 0.15	*p=0.001*

4.3.2.7 Callus formation

There was no difference in the callus formation scores between the four media at establishment (Figure 31.A). After the first subculture, significantly higher callus formation scores were observed on explants cultured on 1MS + 1mg/l BAP (Figure 31.B).

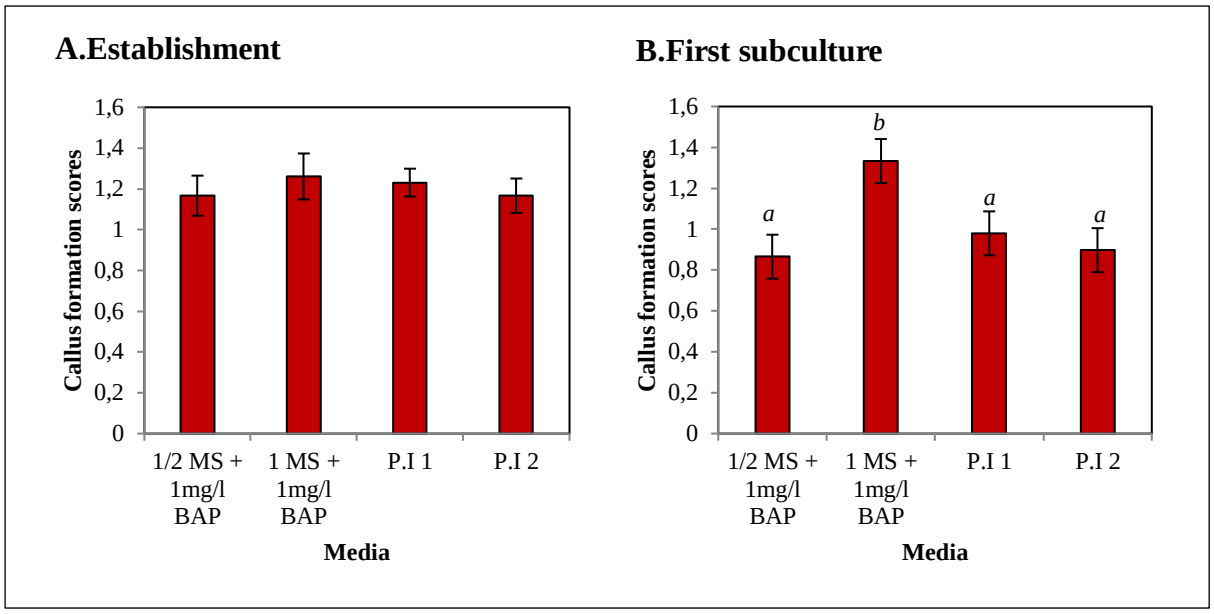


Figure 31. Callus formation score (mean ± SE) of *in vitro* avocado axillary bud explants cultured on four tissue culture media 6 weeks after establishment (A) and the first subculture (B). There was no significant difference in the callus formation scores at establishment (*H(3, 116)= 0.814, p=0.84*), however there was a difference at the first subculture (*H(3,184)= 12.58, p=0.006*).

Callus formation scores decreased from establishment to the first subculture for both P.I. media (Table 27).

Table 27. Callus formation score (mean ± SE) of *in vitro* avocado axillary bud explants cultured on two tissue culture media 6 weeks after establishment and the first subculture.

	Callus formation score (mean ± SE)		
Medium	Establishment	First subculture	*p* values
P.I 1	1.23 ± 0.06	0.97 ± 0.09	*p=0.07*
P.I 2	1.16 ± 0.08	0.89 ± 0.12	*p=0.03*

4.4 DISCUSSION

4.4.1 Stage 1. Establishment of an aseptic culture

After optimising the soil conditions and thus growing healthy mother plants (Chapter Three), a lower level of contamination was observed for the explants cultured on the P.I media than in the initial experiments (Figure 23.A). Additionally, only a single application of the systematic fungicide Ridomil Gold was used, instead of the monthly application which was needed to prepare the mother plants initially. This clearly demonstrated that establishing healthy avocado mother plants was effective in reducing *in vitro* contamination (Barceló-Muñoz and Pliego-Alfaro, 2012; Cooper, 1987).

4.4.2 Stage 2. *In vitro* establishment and multiplication

More than 50% of the explants cultured on both P.I media had pink, light green leaves during establishment (Figure 24.A). This had not been observed with explants cultured on all four media tested in the initial experiments, which had dark green leaves (Figure 24.B). In avocado plants, pink leaves have been associated with both disease and juvenility (Dodds *et al.*, 2001; Wallace, 1958). In other tree species pink leaves have also been associated with an iron deficiency (Van der Salm *et al.*, 1994). Hence, it was possible that the composition of the tissue culture media encouraged abnormal growth. Alternatively, it was possible that different batches of mother plants may have had an impact on the *in vitro* responses of the explants (Greenwood, 1995). This is because the difference in leaf physiology was only observed in the subsequent experiments, in which different mother plants were used. This suggested that using juvenile material from avocado mother plants was not sufficient to develop a reproducible tissue culture system (Castro *et al.*, 1995; Pliego-Alfaro *et al.*, 1987; Ben-Ya'acov, 1976; Gillespie, 1956). Thus, it may be necessary to further pretreat the mother plants, possibly by supplementing them with additional nutrients (Hiti-Bandaralage *et al.*, 2017; Preece, 2008). It may however, also be possible that additional factors relating to the mother plants need to be considered, such the seasonal flush or the proportion of shoot types (proleptic to sylleptic) which were on the plant when the material was harvested (Preece, 2008; von Aderkas and Bonga, 2000; Greenwood, 1995; Murray *et al.*, 1994; Gupta *et al.*, 1991).

4.4.2.1 Tissue browning and death

Tissue browning was observed to be higher in both P.I media, which were supplemented with ascorbic acid, than in the initial media tested (Figure 25.A). Therefore this particular antioxidant may not have any benefit to culture establishment (Figure 25.A). However, because a combination of supplements was tested in both P.I media, it was not possible to attribute tissue browning to this single component. For all the media tested, tissue browning was observed to have been below score 2 (Figure 25.A), and additionally the death of explants was not observed to be significant on any of the media tested at establishment (Figure 26.A). Thus, tissue browning may have been more related to wounding than to media composition (Ahmad *et al.*, 2013; Minocha and Jain, 2000).

4.4.2.2 Hyperhydricity

It is suggested that 2mg/l BAP in both P.I media contributed to explant hyperhydricity (Figure 27) (Barceló-Muñoz and Pliego-Alfaro, 2012; Van Staden *et al.*, 2008; Nel and Kotze, 1982), because *in vitro* hyperhydricity has been attributed to plant growth regulators, particularly exogenous cytokinins (García-Gonzáles *et al.*, 2010; Kataeva *et al.*, 1991). Alternatively, it was possible that nutrient deficiencies in the mother plants had an impact on hyperhydricity *in vitro* (García-Gonzáles *et al.*, 2010; Kataeva *et al.*, 1991), since the explants cultured on both P.I media, which had pink, light green leaves (Figure 24.A), were affected with elevated levels of hyperhydricity at the first subculture (Figure 27.B) (Hazarika, 2006; de la Viña *et al.*, 2001; Ziv, 1991; Daguin and Letouze, 1986). While hyperhydricity appeared to be related to media composition in this study, it would be useful to consider optimising other factors (such as light intensity and container ventilation) which may assist in reducing hyperhydricity in cv. 'Edranol'(Hiti-Bandaralage *et al.*, 2017; Ziv, 1991).

4.4.2.3 Shoot and axillary bud growth

Shoot and axillary bud growth was observed to be significantly higher in the explants cultured on P.I 1 than the other media tested (Figure 28.A and Figure 29.A). Interestingly, P.I 2 was supplemented with the same concentration of PGRs (BAP and GA_3) as the P.I 1 medium but no improvement in growth was observed (Figure 28.A and Figure 29.A). There was no significant difference in the growth of the shoots and axillary buds in explants cultured on P.I 2 when compared with that of the initial experiments (Figure 28.A and Figure 29.A). Due to this, $NaH_2PO_4.2H_2O$, pantothenic acid (Ca-salt) and the higher concentrations of vitamins, as was added in P.I 2, did not appear to benefit growth (Thorp *et al.*, 2008). The higher concentration of BAP was not effective in alleviating apical dominance, as less than 2 shoots per explant developed on both P.I media (Figure 28). Additionally, the number of axillary buds produced per explant for both P.I media were in the range of the initial experiments, which suggested that 2mg/l BAP did not improve growth and development in cv.'Edranol' (Zulfiqar *et al.*, 2009; Nhut *et al.*, 2008; Schaffer *et al.*, 1999; Ahmed *et al.*, 1997; Barringer *et al.*, 1996; Zirari and Lionakis, 1994; Castro *et al.*, 1995; Vega, 1989; Cooper, 1987).

4.4.2.4 Shoot length

Explants cultured on 1MS + 1mg/l BAP produced the tallest shoots at establishment and the first subculture (Figure 30). This medium alone contained the full strength concentration of MS macronutrients and thus it was possible that the elevated nutrient levels were responsible for enhanced shoot growth (Jamshidi *et al.*, 2016; Reed *et al.*, 2013; Kataeva *et al.*, 1991). There appeared to be no benefit to adding GA_3 in both P.I media as shoot length was not enhanced (Figure 30). It was possible, however, that 1mg/l GA_3 may not have been the optimal concentration for shoot extension in cv. 'Edranol' (Bandaralage *et al.*, 2015; Sánchez-Romero *et al.*, 2007; Raharjo and Litz, 2005; del Sol *et al.*, 2000; Vega, 1989; Nel *et al.*, 1983; Young, 1983; Nel and Kotze, 1982). Thus, it would be necessary to test GA_3 in different concentrations to determine if shoot growth and extension could be enhanced.

4.4.2.5 Callus formation

The responses of the explants suggested that callus formation was not related to media but other factors, such as endogenous PGR levels (Barceló-Muñoz and Pliego-Alfaro, 2012; Zulfiqar *et al.*, 2009; Moshkov *et al.*, 2008). This was because callus formation did not differ on the media tested, and remained less than score 2 (Table 5). However, to fully understand the callus responses of the explants it would be more appropriate to quantify the size of the callus instead of using scores related to location. This was because callus formed mainly at the proximal cut end of explants and sometimes filled the entire test tube (Figure 17) (Young, 1983; Schroeder, 1976, 1973).

5 CHAPTER FIVE - General Discussion and Conclusion

The purpose of this study was to develop a system for the multiplication of *in vitro* avocado axillary buds, to be used for the cryo-conservation of this species. The development of such a system for any plant species is a five stage process and the success of each subsequent stage is highly dependent on the performance of the explants in the previous stage. The control of root rot disease was achieved by using well-draining and aerated soils with controlled irrigation. The combination of these culture conditions contributed to plants growing well, without the need for additional fungicides (Barceló-Muñoz and Pliego-Alfaro, 2012; Zulfiqar *et al.*, 2009; Moshkov *et al.*, 2008). As a result, the establishment of healthy mother plants was central to reducing *in vitro* contamination.

While it was suggested in the initial experiments that low and unpredictable responses of avocado explants *in vitro* was related to the slow growth of the avocado mother plants, subsequent experiments done with healthy plant material confirmed that avocado is resistant to tissue culture (Hiti-Bandaralage *et al.*, 2017; Zulfiqar *et al.*, 2009; Barceló-Muñoz *et al.*, 1999; Castro *et al.*, 1995; Pliego-Alfaro and Murashige, 1987). Overall, the behaviour of avocado has been described as recalcitrant and variable responses to *in vitro* propagation have been observed (Castro *et al.*, 1995; Vega, 1989). Furthermore, in other varieties variable regenerative responses have been observed in explants under the same treatment (Castro *et al.*, 1995; Pliego-Alfaro *et al.*, 1987; Ben-Ya'acov, 1976; Gillespie, 1956). Thus, it is possible that slow growth and inconsistent results may be attributed to the physiological state of the mother plants from which material was harvested (Kane, 2016; Barceló-Muñoz and Pliego-Alfaro, 2012; Trippi, 2012; Pliego-Alfaro *et al.*, 1987). This suggests that cv. 'Edranol' mother plants may require further rejuvenation, as was needed with some other varieties (Kane, 2016; Barceló-Muñoz and Pliego-Alfaro, 2012; Trippi, 2012; Zirari and Lionakis, 1994; Schroeder, 1979; Cooper, 1987; Pliego-Alfaro et al., 1987). Additionally, more research is needed to investigate additional physiological factors relating to the mother plants (e.g. endogenous PGR levels) which may influence how the explants subsequently respond to tissue culture.

On all the media tested, there was an increase in tissue browning (Table 7 and Table 21), death (Table 8 and Table 22) and hyperhydricity (Table 9 and Table 23) from establishment to the first subculture. This suggested that the transition from establishment to the first subculture was more stress inducing for the explants than the initial excision from the mother

plants to *in vitro* establishment (Gaspar *et al.*, 2000; Greenwood, 1995). It was possible that the explants developed greater dependence on the exogenous PGRs and nutrients supplied by the tissue culture medium by the first subculture, however, the supplements provided in media tested were insufficient for healthy development (Preece, 2008; Gaspar *et al.*, 2000; Greenwood, 1995). The apparent decrease of any beneficial effect of supplements was in contrast to what was observed in the tissue culture of other plant species where generally explants are generally considered to be highly dependent on exogenous PGR's and nutrients during *in vitro* establishment (Preece, 2008; Gaspar *et al.*, 2000; Greenwood, 1995). It is only after prolonged maintenance in culture that the explants are able to produce PRGs and thus become less dependent on the supplements provided in the tissue culture media (Preece, 2008; Gaspar *et al.*, 2000; Greenwood, 1995). The findings of this study therefore suggested that while the avocado explants did interact with the exogenous PGRs, their growth was primarily directed by endogenous factors (Preece, 2008; Gaspar *et al.*, 2000; Greenwood, 1995).

The focus of this study was to develop an *in vitro* system for the proliferation of axillary buds in avocado cv. 'Edranol', for the purpose of using the buds for the conservation of the species, through cryopreservation. From the six media tested in this study, it appeared that 0.5mg/l BAP resulted in weak explants, as explants cultured on media containing BAP at this concentration were more prone to contamination, browning and death. Additionally, both the P.I media, based on the *P. indica* medium, did not promote the growth and development of healthy explants. Thus, the media containing 1mg/l BAP were considered.

The explants cultured on ½ MS + 1mg/l BAP and 1MS + 1mg/ l BAP performed similarly in terms of contamination (Figure 6), percentage death (Figure 8), hyperhydricity (Figure 10), the number of shoots developed (Figure 12) and the number of axillary buds developed (Figure 14). Only callus formation scores at the first, the second and the third subculture (Figure 18.B-D) and shoot length at the first and the second subculture (Figure 16. B and C) were observed to be significantly higher in 1MS+ 1mg/l BAP compared with ½ MS + 1mg/ BAP. It was observed that ½ MS was associated with an increase in hyperhydricity from establishment to the third subculture (Table 9). Thus, within the scope of this study, the most appropriate medium for axillary bud proliferation was 1MS + 1mg/ l BAP. With this medium, however, the mass multiplication of axillary buds was not achieved, as on average over the four culture periods, the number of shoots developed on this medium ranged between 0.76 ± 0.1 and 1.0 ± 0.1 (Figure 12), while the number of axillary buds ranged

between 3.39 ± 0.2 and 5.04 ± 0.6 per shoot (Figure 14). Additionally, with explants cultured on 1MS + 1mg/ l BAP contamination (4.7-20.58%) (Figure 6), death (5-35%) (Figure 8) and hyperhydricity (18-21%) (Figure 10) were observed. Thus, this medium was appropriate for culture initiation but not for the multiplication of shoot and axillary buds at a level sufficient to allow for further experimentation.

In light of this, it would be useful to further optimise the tissue culture system. The optimal concentration of plant growth regulators may break apical dominance and bring about improved results by generating heathy material. Thus, it would be necessary to experimentally test the plant growth regulators in the tissue culture medium in different concentrations. Additionally, it would be important to optimise the nutrient concentrations to develop a medium which is more suitable for avocado tissue culture than MS medium.

In conclusion, the micropropagation of avocado through tissue culture is not new but has been in process for more than 40 years. However, this technique can still be considered to be in its infancy stages due to strong apical dominance, oxidation and hyperhydricity. As a result, a reproducible system for the proliferation of *in vitro* axillary buds in avocado cv. 'Edranol' was not achieved in this study. While the responses of the material of cv. 'Edranol' to the *in vitro* environment suggested that further optimisation of the micropropagation system is still necessary, research efforts should now be turned to understanding the recalcitrance of avocado vegetative tissues to the *in vitro* environment, in order to generate healthy plant material which could be used to contribute towards the conservation of this species.

5.1 Future recommendations

Based on the findings and conclusions made in this study, a number of recommendations are made. Firstly, avocado mother plants should be further pretreated, possibly through the inclusion of additional nutrients. Secondly, to develop a reproducible tissue culture system, additional factors relating to the mother plants should be determined, such as harvesting time and the developmental stage of the material. Thirdly, to optimise the tissue culture medium for axillary bud multiplication, concentrations of gibberellic acid other than 1mg/l should be tested, and the macro- and micronutrient concentrations in the basal medium should be optimised. Finally, to further understand the responses of the explants to the *in vitro* environment, additional methods should be employed, such as biochemical and microscopical techniques.

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